Cloning and Characterization of E2F6, a novel member of the E2F transcription factor family

by

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Abstract

The E2F family of proteins plays a critical role in the regulation of genes that are essential for progression through the cell-cycle. Based upon sequence homology and functional properties, the E2F group can be subdivided into two subclasses (E2F1, 2, 3 versus E2F4 and 5). The work detailed in this study focuses on the cloning and characterization of a novel E2F family member, E2F6. E2F6 bears some sequence homology to the other E2Fs in the DP dimerization and DNA binding domains, but there is a high degree of divergence outside of those domains. Additionally, although the DP dimerization and DNA binding properties of E2F6 are similar to those of the other E2F family members, it is not regulated by the pocket proteim family and it is unable to activate transcription. Instead, it can act to repress the transcription of E2F responsive genes by countering the activity of the other E2F complexes. Therefore, E2F6 represents a new subclass of the E2F family.

In an effort to more fully understand the mechanism behind the repressive abilities of E2F6, we screened for interacting proteins. RYBP, a member of the mammalian polycomb group, was identified by yeast-two-hybrid analysis as an E2F6-interacting protein. The RYBP-binding domain on E2F6 was highly conserved among all the E2F proteins, but the interaction was nevertheless specific to E2F6. In addition, E2F6 can associate with several other members of the polycomb transcriptional repression complex, including Ring1a, Mel-18, mph1, and the oncogene Bmi1. Moreover, overexpression of E2F6 leads to a similar downregulation of $p19^{ARF}$ as exogenous Bmi1. These findings suggest that the biological properties of E2F6 could be mediated through its ability to recruit the polycomb complex. Finally, the presence of E2F6 in mammalian polycomb complexes sheds insight into how these complexes might bind to DNA.

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Chapter 1

Introduction

Part I: The E2F family

A. The cell division cycle

The cell division cycle is the universal process by which cells duplicate and divide and its regulation is critical for the normal growth and development of all organisms. The most important events in the cell cycle are the replication of the genetic material during S phase and the subsequent partitioning of the DNA during mitosis (M phase). In between these events are two gap phases (G1 and G2) during which cells can grow and prepare for the next step in the cycle. The onset of the S and M phases of the cell cycle is under very rigid control since a cell's survival depends upon these events occurring at the proper time and in the proper order. A lack of precision at either the S or M phase can lead to the genomic instability that is a hallmark of cancer.

In the absence of growth factors, most cells will exit the cell cycle (G0 phase) and remain in that quiescent state until the conditions become favorable for proliferation again (i.e. growth factors are re-added). Upon exposure to mitogenic signals, there is a cascade of events, including several bursts of transcriptional activity, which results in a cell entering the G1 phase of the cell cycle. If conditions remain favorable, the cell passes through a critical moment, called the restriction (R) point, after which the cell is irreversibly committed to undergo DNA replication and cell division (Pardee 1974; Planas-Silva and Weinberg 1997). Since the two primary points of cell cycle control are at the G0/G1 transition and the G1/S transition and one of the hallmarks of cancer cells is a loss of this control, it is important to understand the proteins involved in cell cycle regulation.

B. The cyclins and cyclin dependent kinases

The cyclins, identified by virtue of their cyclical oscillation during the cell cycle, are the primary positive regulators of the cell division cycle (Evans et al. 1983). These proteins provide the regulatory subunits for a set of cyclin dependent kinases (cdks). Together these cyclin/cdk complexes phosphorylate substrates critical for progression through the cell cycle. Distinct combinations of cyclins and kinases are active at particular points in the cell cycle.

The D-type cyclins (D1, D2, and D3) are expressed in early G1 and play an important role in the passage through this phase of the cell cycle. These cyclins are expressed after mitogenic stimulation and form active kinase complexes with either cdk4 or cdk6 (Sherr 1993). Overexpression of cyclin D1 shortens the cell cycle whereas inactivation of cyclin D1 prevents entry into S phase (Quelle et al. 1993; Resnitzky et al. 1994). In these experiments, manipulation of cyclin D1 levels must occur before the restriction point. These results show that cyclin D/cdk complexes must phosphorylate substrates that are critical for passage through G1.

The expression of *cyclin E* and *cyclin A* begins in late G1 and is not dependent on mitogenic signals. Both cyclins bind and regulate the kinase cdk2. The key difference between these two cyclin/cdk complexes is in the timing of their action. Cyclin E/cdk2 activity peaks at the G1/S transition, while the peak of cyclin A/cdk2 activity occurs in S phase. Antibody microinjection experiments show that blocking cyclin E activity results in a G1/S cell cycle arrest, whereas blocking cyclin A activity yields a disruption further along into S phase itself (Pagano et al. 1992b; Ohtsubo et al. 1995). Therefore, cyclins E

and A play key but distinct roles in cell cycle progression through the G1/S transition and S phase respectively.

Given the essential role of cyclin/cdk complexes in the cell cycle, regulation of their enzymatic activity is of vital importance to the cell. One way in which this control is accomplished is through two subgroups of small molecules known as the cdk inhibitors (CDIs). The first group, the Cip/Kip family, consists of $p21^{WAF1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$. These proteins associate with the cyclin D, cyclin E, and cyclin A-containing kinase complexes. For cyclin E/cdk2 and cyclin A/cdk2 complexes, binding by the Cip/Kip family of CDIs leads to an inhibition of kinase activity. In contrast, association of these small molecules with the cyclin D/cdk4/6 kinase complexes is believed to promote kinase activity through a stabilization of the complex (Sherr and Roberts 1999). The second class of inhibitors, the Ink4 proteins, is comprised of $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$ and $p19^{Ink4d}$. These CDIs function more specifically by binding to Cdk4/6 and inhibiting kinase activity by preventing the association of the D-type cyclin. As the expression of the CDIs can be induced under conditions of cellular stress such as serum starvation and DNA damage, these inhibitors perform important regulatory functions that enable cells to halt the cell cycle in response to anti-proliferative cues.

C. The pocket protein family

(i) The retinoblastoma tumor suppressor

The first identified tumor suppressor gene, the retinoblastoma susceptibility gene (*RB-1*) was positionally cloned by virtue of its absence in two human cancers, retinoblastomas and osteosarcomas (Friend et al. 1986; Fung et al. 1987; Lee et al. 1987). Subsequent

studies have revealed that the *RB-1* gene is mutated or absent in at least one-third of all human tumors (Weinberg 1992). Furthermore, proteins produced by small DNA tumor viruses target the retinoblastoma protein product (pRB). Oncoproteins such as adenovirus E1A, SV40 large T antigen and human papillomavirus E7 can bind directly and functionally inactivate pRB (DeCaprio et al. 1988; Whyte et al. 1988; Dyson et al. 1989; Munger et al. 1989). The importance of these interactions is underscored by the fact that the sequences in these viral proteins required for cellular transformation are the same as those required for binding to pRB (Dyson et al. 1992; Nevins 1992).

In untransformed cells, the growth inhibitory functions of pRB are regulated by cell cycle dependent phosphorylation (Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989; Mihara et al. 1989). In quiescent and differentiated cells pRB is found in a hypophosphorylated form (Chen et al. 1989). Additionally, pRB is hypophosphorylated in the early G1 phase of the cell cycle. At the G1/S transition, the cyclin dependent kinase cyclin D/cdk4 initiates the phosphorylation of pRB (Hinds et al. 1992; Ewen et al. 1993). This phosphorylation cascade is completed by the kinase cyclin E/cdk2 and pRB remains in this hyperphosphorylated form throughout the remainder of the cell cycle (Lundberg and Weinberg 1998; Harbour et al. 1999).

There is a multitude of evidence that indicates that hyperphosphorylation of pRB causes its inactivation. First, the viral oncoproteins such as E1A only bind hypophosphorylated pRB. Second, only hypophosphorylated forms of pRB can bind to and inactivate the S phase inducing transcription factor E2F (Chellappan et al. 1991 and see below). Finally, conditions that favor pRB phosphorylation also favor cellular proliferation (Cobrinik et

al. 1992; reviewed in Hatakeyama and Weinberg 1995). These data indicate that pRB is an important cell cycle regulator that acts to block proliferation in the G0/G1 phase.

(ii) p107 and p130

The pocket protein family consists of pRB and the two pRB related proteins, p107 and p130 (reviewed in Beijersbergen and Bernards 1996; Classon and Dyson 2001). Like pRB, p107 and p130 are targeted by viral oncoproteins suggesting that they too play an important part in growth suppression (Ewen et al. 1991; Hannon et al. 1993; Li et al. 1993). In fact, overexpression of p107 or p130 arrests cells in G1, supporting a role for these proteins in cell cycle regulation (Vairo et al. 1995; Zhu et al. 1995). p107 and p130 share considerable sequence homology with pRB especially within the pocket domain that is the defining characteristic of this family (Ewen et al. 1991; Hannon et al. 1993; Li et al. 1993; Mayol et al. 1993). Finally, all the members of the pocket protein family are phosphorylated in a cell cycle dependent fashion (Classon and Dyson 2001).

Although p107 and p130 share many features with pRB, these proteins also possess some unique characteristics. First, their expression patterns are quite distinct. p107 is expressed at high levels in actively dividing cells, whereas p130 is found mainly in cells that have exited the cell cycle (Beijersbergen et al. 1995; Smith et al. 1996). In contrast, pRB is found at moderate levels in both quiescent and cycling cells (Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989). Second, the spacer region in p107 and p130 is larger than in pRB and has been shown to moderate a direct interaction between these pocket proteins and cyclin/cdk complexes (Hu et al. 1992; Ewen et al. 1992). Finally, the E2F proteins constitute a critical downstream target of the pocket proteins; however, each pocket protein binds to a particular subset of the E2F family. Thus,

although the pocket protein family members are highly related, there are distinctions among them that indicate each may have somewhat different functions in cell cycle control.

D. Cloning of the E2F transcription factor

The growth suppressive properties of the pRB family of proteins depend solely on their ability to bind and regulate a transcription factor known as E2F (Nevins 1992; Bartek et al. 1996). E2F activity was originally discovered through its requirement for E1A mediated transactivation of the adenoviral E2 promoter (Kovesdi et al. 1986; Yee et al. 1989). Binding of pRB by the viral protein E1A dissociates E2F from pRB (Bandara and La Thangue 1991). This free E2F species is then complexed with the viral protein E4 orf6/7, which mediates cooperative DNA binding at two sites in the E2 promoter (Huang and Hearing 1989a; Huang and Hearing 1989b; Marton et al. 1990; Neill et al. 1990). E2F DNA binding sites similar to that found in the adenoviral E2 promoter have been identified upstream of a host of cellular genes, primarily those involved in the entry into and subsequent passage through the cell cycle.

When this work was initiated, seven human genes had been identified as components of the E2F transcriptional activity (Beijersbergen and Bernards 1996). On the basis of sequence homology and functional properties, these genes have been divided into two distinct groups, the E2Fs (E2F1 through E2F5) and the DPs (DP1 and DP2). The protein products from these two groups heterodimerize, yielding both high affinity DNA binding complexes and subsequent transcriptional activation(Bandara et al. 1993; Helin et al. 1993; Krek et al. 1993; Wu et al. 1995).

(i) DP proteins

Differentiation regulated transcription factor (DRTF/E2F)-polypeptide-1 (DP1) was originally identified as one of the proteins in a DNA affinity purification of DRTF/E2F activity (Girling et al. 1993). This 429 amino acid protein possesses significant homology to E2F1 in the DNA-binding domain and DP1 mRNA is expressed in a wide variety of cell lines and tissues during murine embryonic development (Girling et al. 1993; Wu et al. 1995; Tevosian et al. 1996; Dagnino et al. 1997). Additionally, DP1 is capable of forming heterodimers with each of the E2F family members.

The second member of the DP family (DP2) was isolated using many different approaches that included a yeast two-hybrid assay, standard library screening techniques and RT-PCR with degenerate oligonucleotides (Ormondroyd et al. 1995; Wu et al. 1995; Zhang and Chellappan 1995; Rogers et al. 1996). DP2 was found to be highly homologous to DP1 and to heterodimerize with the E2F proteins in a similar fashion. However, unlike DP1, DP2 consists of five alternatively spliced mRNA transcripts whose expression varies among cell types and tissues. The significance of these alternative DP2 products is currently not understood.

(ii) E2F proteins

Although the DP subunit is essential for the formation of functional E2F complexes, it is the E2F moiety that mediates the specificity of the complex (Dyson 1998). The five E2F family members that were cloned prior to the onset of this project can be further subdivided into two classes based upon sequence homology and functional properties. The first class contains E2F1, E2F2 and E2F3. These E2Fs are potent transcriptional

activators and hence constitute the "activating" E2Fs. The second class contains E2F4 and E2F5, which are believed to function predominantly in transcriptional repression.

a) The "activating" E2Fs

1) Identification

The founding member of this class, E2F1, was cloned by screening expression libraries for proteins capable of binding to pRB (Helin et al. 1992; Kaelin et al. 1992; Shan et al. 1992). The resulting 437 amino acid protein can bind to DNA in a DP-dependent manner and strongly transactivate E2F responsive promoters. In fact, overexpression of E2F1 can drive quiescent cells into S phase. Furthermore E2F1 interacts specifically with pRB and not with p107 or p130 *in vivo*.

Antibody supershift experiments and other evidence strongly suggested that E2F1 was unable to account for all of the cellular E2F activity. Shortly thereafter, two groups used a low stringency hybridization approach to isolate the cDNAs encoding human E2F2 and E2F3 (Ivey-Hoyle et al. 1993; Lees et al. 1993). These two E2Fs are highly homologous to E2F1 especially in the domains responsible for DNA binding, DP dimerization and pRB binding. Consistent with the significant degree of sequence homology, E2F2 and E2F3 behaved analogously to E2F1 in DNA binding and transactivation assays. In addition, like E2F1, E2F2 and E2F3 bind specifically to pRB and not to its related family members *in vivo*.

When E2F3 was initially cloned, it was noted that if the Northern gels were run for longer times before blotting, E2F3 mRNA resolved into a doublet (Lees et al. 1993). However, the significance of this observation was unexplored until recently when analysis of the E2F3 murine genomic locus revealed that there are in fact two transcripts

expressed (Leone et al. 2000). The first, designated E2F3a, corresponds to the previously characterized E2F3 species. The second transcript, E2F3b, encodes for a protein product that lacks the amino terminal extension present in E2F1, E2F2 and E2F3a, but is otherwise identical to E2F3a in sequence. Furthermore, a previously unrecognized promoter located in the first intron of the E2F3 gene controls its expression (Leone et al. 2000). Unlike the other E2Fs of this subgroup, E2F3b is constitutively expressed. This observation led the authors to propose that E2F3b is regulated differently from the other E2Fs in this class. However, currently it is unknown whether E2F3b participates in the activation or repression of E2F target genes. Therefore, for convenience, I have included E2F3b in the first E2F subgroup.

2) Transcriptional induction of E2F target genes

The "activating E2Fs" can robustly activate transcription in *in vitro* assays (Helin et al. 1993). Additionally, E2F activity was originally found by virtue of its ability to stimulate transcription from the adenoviral E2 promoter (Kovesdi et al. 1986). Given these findings, it is not surprising that E2F-binding sites have been found in a variety of cellular promoters. These genes can be roughly separated into two categories: cell cycle regulators (cyclin E, cyclin A, cdc2, cdc25A, pRB, E2F1) and genes involved in DNA synthesis (DHFR, TK, TS, cdc6, ORC, MCMs). Mutation of the E2F binding sites in the DHFR promoter confirmed that E2F is required to activate expression of this gene during late G1 (Means et al. 1992; Wade et al. 1992). The identification of E2F target genes points to an essential role for the E2F transcription factors in the control of cell cycle progression.

Recently several groups have turned to microarray analysis to more rapidly identify E2F target genes. As predicted based upon the biological activities of E2F proteins, these studies have yielded many genes involved in cell cycle progression, DNA replication, and apoptosis (Ishida et al. 2001; Muller et al. 2001). Many of the genes, however, such as APAF1, were not thought of previously as E2F targets. In addition, a number of genes important for development and differentiation were isolated (Moroni et al. 2001; Muller et al. 2001). Included among these targets were several homeobox (Hox) genes and their polycomb group (PcG) regulators (*Embryonic ectoderm development* and *Enhancer of zeste 2*). These data suggest a possible explanation for the role of E2F proteins in development.

3) Cell cycle entry

A variety of *in vitro* and *in vivo* studies have clearly demonstrated that inappropriate release of free E2F1 can activate DNA synthesis in cells that would otherwise be growth arrested. Overexpression of E2F1 using recombinant retroviruses, microinjection techniques and stable transfections has shown that this E2F family member is capable of inducing cell cycle progression (Johnson et al. 1993; Qin et al. 1994; Shan and Lee 1994; Kowalik et al. 1995; Lukas et al. 1996; DeGregori et al. 1997). E2F1 mutants defective in either DNA binding or transactivation activities were unable to drive quiescent cells into S phase (Johnson et al. 1993). Similar to E2F1, overexpression of either E2F2 or E2F3a can induce DNA synthesis (DeGregori et al. 1997; Vigo et al. 1999). *In vivo* experiments confirm that the members of the first subgroup of the E2F family play a critical role in cell cycle progression. Removal of either E2F1 or E2F3 in the context of

an RB deficiency revealed that both proteins contribute to the inappropriate S phase entry observed in $RB^{-/-}$ mice (Tsai et al. 1998; Ziebold et al. 2001).

Overexpression of E2F1 can override a number of growth arrest signals including the effects of CKIs (p16, p21 and p27) and some growth factors (TGFβ) (DeGregori et al. 1995; Schwarz et al. 1995; Mann and Jones 1996). Consistent with these observations, E2F1 and DP1 can cooperate with oncogenic ras in the transformation of primary cells (Johnson et al. 1994a; Jooss et al. 1995). Additionally, expression of E2F2 and E2F3a in cells produced a loss of contact inhibition and allowed for growth in a soft agar medium, two hallmarks of tumorigenic cells (Singh et al. 1994; Xu et al. 1995). Therefore, E2F1, E2F2 and E2F3a each play an important role in cell cycle progression.

4) Apoptosis

In addition to driving cells into S phase, deregulated E2F activity can also cause apoptosis in both cell culture and mouse model systems (Qin et al. 1994; Shan and Lee 1994; Wu and Levine 1994; Hiebert et al. 1995; Kowalik et al. 1995; Tsai et al. 1998; Ziebold et al. 2001). Whereas there is little dispute that overexpression of E2F activity leads to apoptosis, there is extensive debate about both the mechanism through which the apoptosis is initiated and which E2F family members can trigger it.

E2F induced apoptosis can occur through both p53 dependent (Qin et al. 1994; Wu and Levine 1994; Hiebert et al. 1995) and p53 independent (Hsieh et al. 1997; Phillips et al. 1997; Phillips et al. 1999) mechanisms. A potential link between E2F and p53 mediated apoptosis comes from the observation that $p19^{ARF}$ expression can be induced by E2F1 (DeGregori et al. 1997; Bates et al. 1998). An increase in $p19^{ARF}$ protein leads to an increase in the stabilization of the p53 protein and a concomitant increase in p53

transactivation (Kamijo et al. 1998; Pomerantz et al. 1998; Stott et al. 1998; Zhang et al. 1998). Recent reports demonstrating that the p53 family member, p73, can be induced by E2F1 activity provide a possible explanation for the p53-independent apoptosis caused by E2F (Irwin et al. 2000; Lissy et al. 2000; Stiewe and Putzer 2000). Although both of these links are reported to be direct, the relative contributions made by $p19^{ARF}$ and p73 to E2F-induced apoptosis is still under investigation.

There is also some question as to which E2F family members can cause apoptosis. DeGregori *et al* observed that only E2F1 and not E2F2 or E2F3 could induce an apoptotic response in their cell-based assay (DeGregori et al. 1997). However, subsequent cell culture experiments demonstrated that ectopic expression of E2F1, 2, and 3 could all trigger apoptosis (Vigo et al. 1999). Mouse model experiments examining the contribution of specific E2Fs to the apoptosis seen in Rb mutant embryos showed that both E2F1 and E2F3 could contribute to the cell death observed in $Rb^{-/-}$ mice (Tsai et al. 1998; Ziebold et al. 2001). Despite these results, the debate has continued recently with the publication of a paper showing that c-myc can induce apoptosis efficiently in the absence of E2F2 and E2F3, but not E2F1 (Leone et al. 2001). These results are most likely specific to the adenoviral delivery system used by this group since experiments conducted in our lab have failed to reveal any difference in c-myc induced apoptosis between $E2f1^{-/-}$ and $E2f3^{-/-}$ MEFs (A. Aslanian unpubl. data).

5) Regulation of E2F activity by pocket protein binding

The appearance of the "activating" E2Fs is regulated at the level of transcription. mRNA levels for E2Fs 1, 2, and 3a rise significantly in late G1, peaking at the G1/S transition. This increase in transcription is regulated at least partially through the E2F

binding sites present in the promoters of E2F genes (Hsiao et al. 1994; Johnson et al. 1994b; Neuman et al. 1994). In addition to being controlled at the level of transcription, the E2F proteins are also subject to many other levels of regulation.

The E2F family members are critical downstream targets of the pocket protein family. E2F1 was initially cloned by virtue of its association with pRB and E2F2 and E2F3 have subsequently been shown to bind specifically to pRB (Lees et al. 1993). Mapping studies have identified a highly conserved 18 amino acid stretch, embedded in the C-terminal transactivation domain of the E2Fs, as necessary for the pocket protein/E2F interaction (Helin et al. 1993). Additionally, heterodimerization with DP is required for the E2F proteins to interact with the pocket proteins (Helin et al. 1993; Krek et al. 1993).

When pocket proteins bind E2F/DP complexes, these trimolecular complexes are still competent to bind DNA (Krek et al. 1993). However, since the pocket protein-binding domain is contained with the E2F transactivation domain, association with the pRB family severely inhibits the ability of the E2Fs to enhance transcription (Helin et al. 1993; Ginsberg et al. 1994; Hijmans et al. 1995; Vairo et al. 1995). Moreover, these pocket protein/E2F/DP complexes can recruit additional factors to modify the local chromatin structure. In this way these complexes can also participate in the active repression of E2F target genes. Cell cycle dependent phosphorylation of pRB releases free E2F/DP complexes and allows them to stimulate transcription. Thus, E2F transcriptional activity is under strict cell cycle dependent control by the pocket proteins.

6) Regulation of E2F by phosphorylation

A conserved motif in the N-termini of E2F1, 2 and 3 mediates an interaction between the E2F protein and the cyclin A/cdk2 kinase (Mudryj et al. 1991; Devoto et al. 1992;

Pagano et al. 1992a; Krek et al. 1994; Xu et al. 1994). Normally, cyclin A/cdk2 kinases bind E2F and phosphorylate the DP subunit. This phosphorylation serves to inhibit E2F DNA binding activity after the activation of E2F target genes in S phase (Dynlacht et al. 1994b; Xu et al. 1994; Krek et al. 1995). Mutation of this cyclin binding site in E2F1 results in more stable DNA binding, a concomitant transient S-phase arrest, and eventually apoptosis (Krek et al. 1995). Thus, direct binding of cyclin A/cdk2 to E2F and subsequent phosphorylation of DP are required for proper progression through the cell cycle.

Increased phosphorylation of the DP protein and downregulation of E2F/DP DNA binding activity has also been correlated with cell cycle exit in a model of adipocyte differentiation (Altiok et al. 1997). In this system, the increase in DP phosphorylation was believed to result from the downregulation of a protein phosphatase (PP2A). These data showed that phosphorylation of E2F/DP complexes may also be critical for cell cycle withdrawal.

7) Regulation of E2F by acetylation

The transcriptional coactivator p300 and the highly related CREB binding protein (CBP) are believed to stimulate transcriptional activation through their associated histone acetyl transferase (HAT) activity (Bannister and Kouzarides 1996; Ogryzko et al. 1996; reviewed in Goodman and Smolik 2000). Although the first identified targets for CBP/p300 acetylation were the histones, it has become apparent that other transcription factors, such as GATA-1, p53, HMG-I(Y), and TFIIF can be modified as well (Gu and Roeder 1997; Imhof et al. 1997; Boyes et al. 1998; Munshi et al. 1998; Zhang and Bieker 1998). E2F1 can associate with p300 and CBP through sequences present in its C-

terminal transactivation domain (Trouche et al. 1996). This association leads to an increase in the ability of E2F1/DP1 heterodimers to activate transcription (Trouche et al. 1996). Recently a potential mechanism for this CBP/p300 stimulation was elucidated. CBP/p300 and an associated factor, P/CAF, were found to be capable of acetylating E2F1, 2, and 3 in vitro (Martinez-Balbas et al. 2000; Marzio et al. 2000). Further analysis revealed that E2F1 was in fact acetylated *in vivo* (Martinez-Balbas et al. 2000). These acetylations occurred on conserved lysine residues adjacent to the DNA binding domain and served to enhance both the DNA binding and transactivation potential of the E2F/DP complex (Martinez-Balbas et al. 2000; Marzio et al. 2000). These data reveal another reversible modification that plays a role in the regulatory network of E2F activity.

8) Regulation of E2F by ubiquitin mediated proteolysis

Regulated degradation may also play an important role in the control of E2F activity. Targeting of proteins for destruction by the 26S proteasome requires the addition of a polyubiquitin chain. This conjugation requires the activity of three enzymes: an ubiquitin activating enzyme, E1, an ubiquitin-conjugating enzyme, E2, and an ubiquitin protein ligase, E3 (reviewed in Hochstrasser 1996). In overexpression experiments, E2F1 was shown to be an unstable protein and subject to degradation by the ubiquitin dependent proteasome pathway (Hateboer et al. 1996; Hofmann et al. 1996). Deletion mutagenesis mapped the region responsible for the instability to the C-terminal pocket protein-binding domain of the E2F proteins (Hateboer et al. 1996). Consistent with the mapping data, association with pRB afforded E2F1 significant protection from degradation (Hateboer et al. 1996; Hofmann et al. 1996). Endogenous E2F1 has also

been shown to be a short-lived protein that is greatly stabilized under conditions where pRB is hypophosphorylated (i.e. differentiation, cell cycle exit and post-irradiation) (Martelli and Livingston 1999). This regulated degradation could exist to ensure that free E2F does not persist for lengthy periods of time in the cell, where it can lead to unscheduled S phase entry or apoptosis.

The E3 ubiquitin protein ligase is thought to play a critical role in mediating substrate recognition (Hershko and Ciechanover 1998). The Skp1-Cullin-F-box (SCF) complex represents one class of E3 enzymes (Bai et al. 1996). This ubiquitin ligase utilizes sequences in the F-box protein to recruit specific targets that are then polyubiquitinated by the associated E2 enzyme (Patton et al. 1998). In co-transfection experiments, E2F1 has been shown to interact with the F-box protein $p45^{SKP2}$ (Marti et al. 1999). The extreme N-terminal 41 amino acids of E2F1 mediate this interaction and, since this region is conserved with E2F2 and E2F3, could potentially mediate a similar interaction between Skp2 and these E2Fs as well. Mutation of these amino acids resulted in the stabilization of E2F1 and a decrease in ubiquitination of the protein (Marti et al. 1999). These results suggest a possible mechanism for the regulated destruction of the E2F proteins in the S/G2 phase of the cell cycle. However, E2F1 protein levels are normal in cells derived from Skp2 deficient mice (Nakayama et al. 2000), indicating that there are additional pathways for the degradation of the E2Fs.

Recently a second potential mechanism of E2F degradation has been proposed. Overexpression of $p19^{ARF}$ was shown to decrease specifically the stability of E2F1, E2F2 and E2F3 in a proteasome dependent fashion (Martelli et al. 2001). In agreement with these findings, $p19^{ARF}$ was found to interact with E2Fs1-3. Previously, $p19^{ARF}$ was

shown to sequester the MDM2 protein in the nucleolus, inhibiting its ability to facilitate the export and subsequent degradation of the tumor suppressor p53 (Zhang et al. 1998; Tao and Levine 1999; Weber et al. 1999; Zhang and Xiong 1999). Ectopic expression of $p19^{ARF}$ was also able to localize E2F1 into the nucleolus (Martelli et al. 2001). The biological consequence of this latter function is unclear, however. Is the nucleolar localization of E2F just a precursor to the degradation or does it serve some other purpose? In any event, these data provide a potential link between E2F stability and $p19^{ARF}$; the exact nature of this link is still not well understood.

9) Mouse models

An important question in the E2F field is whether the different E2Fs regulate distinct target genes or if they have overlapping functions. Even if the E2Fs are functionally redundant, one might still anticipate, based upon the expression patterns, different tissue-specific requirements for individual E2F family members. Mice deficient for many of the E2F family members have been generated in an attempt to address these questions.

E2F1, the first cloned E2F, was also the initial E2F to be knocked out in mice. *E2f1*$^{-/-}$ mice were found to be viable and fertile (Field et al. 1996; Yamasaki et al. 1996). Consistent with this observation, MEFs derived from these mice display normal cell cycle kinetics and E2F target regulation (see Appendix C). There are, however, some tissue specific abnormalities found in these mice. Nuclei from the cells of the pancreas and salivary glands of the *E2f1*$^{-/-}$ mice are abnormally large and in many cases binucleate. *E2f1*$^{-/-}$ males experienced testicular atrophy between 9 and 12 months of age. In addition, *E2f1*$^{-/-}$ mice possess an excess of T cells (Field et al. 1996). This defect has been shown subsequently to result from the failure of autoreactive *E2f1*$^{-/-}$ T cells to undergo negative

selection (Zhu et al. 1999; Garcia et al. 2000). Finally, and most surprising, the E2F1 deficient mice exhibit a broad spectrum of tumors arising between 8 and 18 months of age.

Another member of the "activating" E2F subclass, E2F3, has also been targeted in mice. This knockout mouse model deletes both E2F3a and E2F3b. *E2f3*$^{-/-}$ mice generated in the pure 129/Sv background are inviable, but in the mixed C57BL/6-129/Sv background 25% of the expected number reached adulthood (J. Cloud et al unpubl. data). The survivors develop dilated cardiomyopathy and died prematurely (average 14.3 months versus 22.3 months) with atrial thrombi and congestive heart failure (J. Cloud et al unpubl. data). Significantly, these mice showed no signs of an increase in tumor incidence. MEFs isolated from these animals exhibit a severe proliferation defect and downregulation of most known E2F target genes (see Appendix C). Together the phenotypes of the E2F1 and E2F3 deficient mice suggest that these two E2F family members have distinct roles *in vivo*.

Whereas the individual knockout mice indicate that E2F1 and E2F3 have distinct roles, crosses between these two genotypes reveal that these proteins have overlapping roles as well. *E2f1*$^{-/-}$;*E2f3*$^{-/-}$ mice die early in embryogenesis (C. Rogers unpubl. data). Double heterozygous mice display a combination of the phenotypes observed in either of the single knockout mice. They exhibit testicular atrophy and the same tumor spectrum as the *E2f1*$^{-/-}$ mice and an E2F3$^{-/-}$ like dilated cardiomyopathy. These data strongly suggest that although E2F1 and E2F3 may still have some distinct roles, they are overlapping in their requirements for development.

b) The "repressive" E2Fs

1) Identification

The second subclass of the E2F family contains E2F4 and E2F5. E2F4 was originally isolated by both degenerate oligonucleotide PCR and expression library screening (Beijersbergen et al. 1994; Ginsberg et al. 1994). This 413 amino acid protein, like the previously described E2Fs, requires association with a DP protein for high affinity DNA binding activity. E2F4 binds pRB; however, unlike the activating E2Fs, E2F4 also binds the pRB related proteins p107 and p130. Also in contrast to the "activating" E2Fs, E2F4 is a poor transcriptional activator and cannot drive quiescent cells into the cell cycle.

E2F5 was cloned in a yeast two-hybrid assay as an interacting protein of both p107 (Hijmans et al. 1995) and p130 (Sardet et al. 1995). The 346 amino acid protein has more similarity to E2F4 than the other E2Fs, associates specifically with p130 *in vivo*, and is highly reminiscent of E2F4 in its inability to activate transcription. These functional properties of the second subgroup of the E2F family have led to the hypothesis that these E2F proteins will be more involved in regulating the events of early G0/G1 than the events of the G1/S transition.

2) Regulation of E2F by subcellular localization

In contrast to the "activating" E2Fs, E2F4 and E2F5 are constitutively expressed (Moberg et al. 1996). E2F4 represents the majority of the total E2F DNA binding activity in the cell and its levels do not change significantly across the cell cycle (Moberg et al. 1996). However, the appearance of free E2F4 complexes does not coincide with the activation of E2F target genes (Moberg et al. 1996). These findings led to the observation that E2F4 undergoes substantial changes in its subcellular localization across

the cell cycle whereas E2Fs1, 2 and 3 are constitutively nuclear (Lindeman et al. 1997; Muller et al. 1997; Verona et al. 1997). In quiescent cells and in early G1, free E2F4 is found exclusively in the cytoplasm while the pRB-E2F4 species was equally distributed in both the nucleus and the cytoplasm (Magae et al. 1996; Lindeman et al. 1997; Muller et al. 1997; Verona et al. 1997). As cells progressed through the cell cycle, pRB was phosphorylated, releasing E2F4 and this free E2F4 protein became more cytoplasmically localized (Verona et al. 1997). Since E2F4 is only present in the nucleus when it is bound to pRB, these results pointed to a model in which E2F4 complexes would play an important role in the repression, instead of the activation, of E2F target genes.

E2Fs1-3 localize to the nucleus through the use of a canonical basic nuclear localization signal (NLS) located near the N-terminus (Muller et al. 1997; Verona et al. 1997). E2F4 lacks an NLS. Furthermore, it has recently been shown that E2F4 is exported from the nucleus in a CRM1 dependent fashion (Gaubatz et al. 2001). This export function is mediated by the presence of two leucine/isoleucine rich hydrophobic nuclear export signals (NES) in the E2F4 protein. The observation that a $p16^{ink4a}$ induced cell cycle block was much less effective under conditions where E2F4 was driven into the cytoplasm demonstrates a critical role for the regulation of the localization of E2F4 complexes in cell cycle withdrawal (Gaubatz et al. 2001).

3) Repression of E2F target genes

Despite the original identification of E2F as an activator of transcription, E2F complexes also play an important role in transcriptional repression. As noted above, pRB/E2F/DP complexes are still competent to bind DNA. Therefore, E2F/DP heterodimers can recruit pocket proteins to the promoters of E2F target genes and repress

gene transcription (Weintraub et al. 1992). Also, mutation of the E2F binding sites upstream of E2F target genes, such as *B-myb*, *cdc2* and *E2F1* for example, results in an increase in the activity from these promoters (Dalton 1992; Lam and Watson 1993; Hsiao et al. 1994). *In vivo* footprinting experiments further demonstrate that the E2F sites in the *B-myb*, *cdc2* and *cyclin A2* promoters are occupied only in G0/G1 (Tommasi and Pfeifer 1995; Huet et al. 1996; Zwicker et al. 1996).

Since the pocket protein-binding domain on the E2Fs is contained within the transactivation domain, pocket protein mediated repression could just be a case of interference with E2F activity. However, more recent data suggests that pRB can participate in a more active and more global transcriptional repression reviewed in (Zhang and Dean 2001). For example, pRB can associate with histone deacetylase enzymes (HDACs) and recruit them to promoters (Brehm et al. 1998; Luo et al. 1998; Chen and Wang 2000; Dahiya et al. 2000). The activity of these enzymes is correlated strongly with transcriptional repression. Additionally pRB can interact with the histone methyltransferase SUV39H1 and recruit it to the promoters of target genes (Nielsen et al. 2001). SUV39H1 methylates lysine 9 in histone H3, creating a binding site for the heterochromatin-associated protein HP1 (Bannister et al. 2001; Lachner et al. 2001). Through its chromodomain, HP1 can interact with methylated lysines on histone proteins and it is through this mechanism that HP1 is believed to spread silencing across the chromosome (Bannister et al. 2001). The association between pRB and SUV39H1 is one way in which this methyltransferase can be recruited to different target genes.

Although these *in vitro* experiments clearly demonstrate a role for pocket protein/E2F/DP complexes in the repression of E2F target genes, there is still

considerable debate over when these complexes act. Mouse embryonic fibroblasts (MEFs) derived from mice deficient for both p107 and p130 exhibited de-repression of some E2F target genes including B-myb, E2F1, cdc2 and cyclin A2 (Hurford et al. 1997). These results have been used to propose a model in which E2F/pocket protein complexes play a critical role in the repression of E2F targets during the early phases of the cell cycle. However, recent data has led to a revising of the above model. Mice deficient for E2F4, E2F5 or E2F4;E2F5 have been generated and cells isolated from these mice enter the cell cycle with wild-type kinetics and display no apparent cell cycle dependent de-repression of E2F target genes (Lindeman et al. 1998; Gaubatz et al. 2000; Humbert et al. 2000). Instead, these animals possess numerous defects in terminal differentiation. Taken together, these data suggest a model whereby pocket protein/E2F/DP complexes play a critical role in the repression of E2F target genes during the terminal cell cycle exit associated with differentiation.

4) Mouse models

Knockout mice have also been generated for the E2F proteins belonging to the second subgroup. *E2f4*$^{-/-}$ mice exhibit craniofacial defects that lead to an increased susceptibility to opportunistic bacterial infections and eventually death (Humbert et al. 2000). Loss of E2F4 also leads to cell type specific deficiencies in terminal differentiation. The most severely affected cell lineage is the erythrocyte, with the mice showing a high number of incompletely enucleated red blood cells. Additionally, another group has reported that the *E2f4*$^{-/-}$ mice have problems with the differentiation of gut tissue and other cell types of the hematopoeitic system (Rempel et al. 2000). MEFs isolated from *E2f4*$^{-/-}$ embryos displayed normal cell cycle kinetics and E2F target gene expression, strongly suggesting

that E2F4 was dispensable for the control of cellular proliferation (Humbert et al. 2000; Rempel et al. 2000).

E2f5$^{-/-}$ mice die several weeks after birth with hydrocephalus caused by the excessive secretion of cerebral spinal fluid. *E2f5*$^{-/-}$ MEFs, like the E2F4 deficient cells, lack a cell cycle defect (Lindeman et al. 1998). Additionally, E2F4;E2F5 double homozygotes die around day 15 of gestation (Gaubatz et al. 2000). Taken together, data from these mutant mouse strains shows that the members of the second E2F subgroup play a critical role in the terminal differentiation program of a variety of cell types.

(iii) E2F proteins in other species

Given the possibility of functional redundancy in mammalian systems, many researchers have examined the role of E2F in other species, where not as many family members may exist. The fully sequenced *Drosophila melanogaster* genome contains two E2F genes (*de2f1* and *de2f2*) and a single DP gene (*dDP*). As in mammalian cells, dE2F1 and dDP form heterodimers that are competent to bind DNA and activate transcription (Dynlacht et al. 1994a; Ohtani and Nevins 1994). Overexpression of dE2F1/dDP drives quiescent cells into the cell cycle and can stimulate apoptosis (Asano et al. 1996; Du et al. 1996). Although the phenotypes of loss of function alleles of *de2f1* and *dDP* are not completely identical, these mutant flies demonstrate an essential role for dE2F1/dDP in activating the G1/S transcriptional program (Royzman et al. 1997).

Recently a second *Drosophila* E2F family member has been cloned (Sawado et al. 1998). While dE2F2 can also bind dDP, these complexes appear to play a role in the repression of target genes (Sawado et al. 1998; Frolov et al. 2001). In fact, flies mutant for dE2F2 have shown that this protein normally functions to antagonize dE2F1 in

Drosophila development (Frolov et al. 2001). However, the specific mechanism of dE2F2-mediated repression is not understood.

E2F and DP proteins have also been isolated in other species. Identification and characterization of a *C. elegans* E2F and DP homolog revealed a role for these proteins in transcriptional repression similar to mammalian E2F4 complexes (Ceol and Horvitz 2001). The *C. elegans* system allows for the dissection of this repression without the presence of additional E2F5 activity.

The study of E2Fs in other species has also provided evidence for novel functions. Xenopus E2F (xE2F) was shown to be involved in the formation of the anterior-posterior axis through the regulation of Hox genes (Suzuki and Hemmati-Brivanlou 2000). In conclusion, the characterization of E2F and DP proteins from non-mammalian species has expanded our knowledge of how these proteins function *in vivo*.

Biochemical studies using antibodies specific for each of the different mammalian E2F family members (1-5) demonstrated that the known E2Fs were unable to account for all of the E2F-DP DNA binding activity. In this study, Chapter 2 details the cloning and initial characterization of a novel member of the E2F family, E2F6. In Chapter 3, using a yeast two-hybrid assay to identify E2F6 interacting proteins, we demonstrate that RYBP is an E2F6 interacting protein. Furthermore, we show that E2F6 can associate with many known members of the Bmi1-containing polycomb complex and thereby gain insight into the mechanism by which E2F6 represses transcription. Finally, in Chapter 4, we describe our recent work aimed at understanding the biological role of the E2F6-containing polycomb complexes.

PART 2: The Polycomb Group

I. Polycomb proteins in *Drosophila*

A. Identification of homeotic mutants in *Drosophila*

Cells become committed to differentiate along specific developmental pathways well before the appearance of structures characteristic of the particular tissue. The fruit fly, *Drosophila melanogaster*, has long been a favorite model organism for studying the phenomena of cell fate determination. In *Drosophila*, the determination of some adult structures begins during embryogenesis in the primordial sac-like clusters known as imaginal discs. However, the imaginal discs do not terminally differentiate until the pupal stage (reviewed in Cohen 1993). Once determined, the imaginal disc cells maintain their fate despite subsequent cell divisions. In fact, the imaginal disc cells fail to change their identity even when transplanted to different regions of the embryo (Simcox and Sang 1983). Therefore, a mechanism must exist not only for the initiation of cell determination, but the maintenance as well, even in actively proliferating cells.

William Bateson originally discovered homeotic transformations at the end of the 19th century (Bateson 1894). He coined the term homeosis to describe mutations in which one body part had been transformed into another. Homeotic loss of function mutations were subsequently identified in *Drosophila*. Two of the mutants initially isolated were *bithorax* and *antennapedia*. Originally identified by Calvin Bridges in 1915, the *bithorax* mutant fly has an extra pair of wings due to the transformation of its third thoracic segment into the second thoracic segment (Lewis, 1963; Lewis, 1978). In the *Antennapedia* mutant fly, the antennae are transformed into leg-like structures (Kaufman

et al. 1990; Plaza et al. 2001). The mutated genes responsible for these phenotypes, the homeotic (Hox) genes, are located in two large clusters in the *Drosophila* genome, the bithorax complex (BX-C) and the antennapedia complex (ANT-C) (reviewed in Lawrence and Morata 1994). Classic experiments by E.B. Lewis further dissected the mutations in the BX-C genes and demonstrated that the normal function of each member of the BX-C is restricted to a specific part of the developing fly embryo (Lewis 1963, reviewed in Lawrence and Morata 1994). The ANT-C genes function in a similar manner, except that their expression and site of action is more anterior than that of the BX-C (Kaufman et al. 1990).

Mutagenesis studies revealed that the BX-C is composed of three homeotic genes, *Ultrabithorax*, *Abdominal-A*, and *Abdominal-B* while the ANT-C consists of five genes, *labial*, *proboscipedia*, *Deformed*, *Sex combs reduced*, and *Antennapedia*. These Hox genes define a family of transcription factors that is characterized by a conserved 60 amino acid DNA-binding domain known as the homeobox. *In vitro* DNA binding experiments demonstrated that the majority of homeobox proteins recognize the sequence 5'-TAAT-3' (Laughon 1991). The small size of this consensus sequence is somewhat surprising given the dramatically different effects Hox gene mutations have on *Drosophila* development. One potential explanation is the existence of cofactors such as *extradenticle* (Peifer and Wieschaus 1990). Association of homeobox proteins with their dimeric partners might alter or refine the DNA binding specificity of these proteins beyond the simple 5'-TAAT-3'and thereby allow the heterodimer to perform more specific or distinct functions.

As described above, maintenance of precise segment-specific expression of the two clusters of homeotic genes is absolutely required for the proper determination of imaginal disc cells (Duncan 1987; Kaufman et al. 1990; Laney and Biggin 1992). Either loss of expression or ectopic expression of the Hox genes in *Drosophila* can have severe consequences for the development of the adult insect, as homeotic mutations in *antennapedia* or *bithorax* clearly demonstrated. Interestingly, even inactivating Hox expression late in development can alter the determined state of particular cell types (Lewis 1963, Morata and Garcia-Bellido 1976; Sanchez-Herrero et al. 1985a; Sanchez-Herrero et al. 1985b).

Hox gene misregulation can also result in more subtle phenotypes. One example is illustrated by changes in expression of the *sex combs reduced* (*scr*) gene. Ectopic *scr* expression causes the appearance of dark, thick bristles (sex combs) on all three pairs of legs in the adult male fly, whereas loss of *scr* expression leads to an absence of the sex combs altogether (Pattatucci and Kaufman 1991; Pattatucci et al. 1991; reviewed in Kennison 1995). Notwithstanding the degree of developmental consequences, maintaining proper Hox gene expression is critical to the development and survival of the organism.

B. Regulation of early Hox gene expression

In early fly development, each homeotic gene is expressed in a spatially restricted domain along the anterior/posterior axis (reviewed in Simon 1995). This expression initiates in the syncytial blastoderm about two hours into embryogenesis and is then stably maintained throughout the course of development. Two classes of transcription

factors set up the initial patterns of Hox expression: the gap and pair-rule proteins. The distribution of most gap and pair-rule gene products is also spatially restricted in the early embryo and maternally provided factors such as bicoid are responsible for establishing these patterns (Lawrence 1992). Analysis of homeotic gene promoters has revealed that, in general, gap genes are repressors and pair-rule genes are activators of homeotic gene transcription. Proper homeotic gene expression is the result of a competition between these two classes of DNA-binding factors for common or overlapping binding sites (Muller and Bienz 1992; Zhang and Bienz 1992; Qian et al. 1993; Shimell et al. 1994).

Ultrabithorax (*ubx*) is a well-studied example that illustrates how Hox gene expression is regulated in the early *Drosophila* embryo. Pair-rule gene products such as fushi tarazu and even-skipped can bind directly to enhancers in the *ubx* promoter and activate transcription of *ubx* (Muller and Bienz 1992; Busturia and Bienz 1993; Qian et al. 1993). However, Ubx is not expressed in all regions that *fushi tarazu* and *even-skipped* transcripts are found. In the regions where Ubx is not expressed, gap gene products such as hunchback and Kruppel act as repressors by binding to promoter elements that overlap the fushi tarazu/even-skipped binding sites (Qian et al. 1991; reviewed in Bienz and Muller 1995). The consequences of this competition for binding sites are determined by the local concentrations of the factors involved and result in the sharp boundaries of Hox gene expression observed.

About four hours into embryogenesis, expression of the gap and pair-rule genes disappears and remains absent throughout the subsequent larval development. However, Hox gene expression is still properly regulated. Therefore, other regulators of homeotic gene expression must act to regulate Hox gene expression later in development.

C. Identification of the regulators of Hox gene expression

Genes that control the expression of Hox genes later in development have been identified. These late regulators belong to two classes of genes: the polycomb group (PcG) and the trithorax group (trxG). PcG proteins are responsible for the maintaining the Hox genes in a repressed state, while the trxG proteins ensure that Hox gene expression continues in those cells where the Hox genes are initially transcribed. Each of these groups contains numerous members that are vital for maintaining proper Hox gene expression patterns.

a) *Drosophila* polycomb proteins

In 1947, P. Lewis identified a dominant mutation in a gene he called *Polycomb* (*Pc*) (Lindsley and Zimm 1992). This mutant resulted in the presence of sex combs on the second and third legs of male flies, instead of just on the first leg. The gene mutated in these flies was subsequently cloned and constitutes the founding member of a growing family of proteins. From this initial mutant, the polycomb group has been defined as genes that, when mutated, cause phenotypes that "resemble weak Pc mutations in both their dominant adult and recessive embryonic phenotypes" (Jurgens 1985). Using a screen based on the observation that mutations in polycomb group genes can synergize and produce even more pronounced phenotypes, Jurgens hypothesized that there are approximately 40 polycomb group genes present in the *Drosophila* genome. At this time, however, only 14 PcG genes have been cloned. These genes, including *Polycomb,* function together in the maintenance of Hox gene repression.

(i) Polycomb

The *Polycomb* gene encodes a 390 amino acid protein that is uniformly expressed during *Drosophila* development. Homozygous mutant flies die at the end of embryogenesis with posterior transformations of all segments, reminiscent of a general deregulation in homeotic gene expression (Lewis 1978; Denell and Frederick 1983; Sato and Denell 1985; Tiong and Russell 1990). Immunostaining of salivary gland polytene chromosomes revealed that the Pc protein associates with ~60-100 discrete sites, including the BX-C and ANT-C regions (Zink and Paro 1989; Franke et al. 1992). Perhaps the most striking feature of the Pc protein is a 37 amino acid stretch that is highly homologous (65% identity) to the chromodomain, a motif originally found in a suppressor of position effect variegation Su(var) 205 (Paro and Hogness 1991). Mutations in the chromodomain of Pc eliminate its ability to associate with the polytene chromosomes *in vivo* (Messmer et al. 1992). Even though the chromodomain is the only recognizable motif present in the Pc protein, fusions between regions of Pc and a heterologous DNA binding domain (Gal4-Pc) showed that the C-terminus plays an essential role in the ability of Pc to repress transcription.

(ii) Polycomb-like

The phenotype of *Polycomblike* (*Pcl*) mutant flies is, as its name implies, very similar to those of *Pc* mutant flies with the second and third legs of males resembling the first (Duncan 1982; Breen and Duncan 1986). In addition, Hox genes of the BX-C and the ANT-C are misregulated in *Pcl* mutant flies (Duncan 1982). As was the case with the other PcG proteins, Pcl localizes to ~100 sites on polytene chromosomes and double labeling experiments revealed that these sites completely overlap with those of Pc (Lonie et al. 1994). The 857 amino acid Pcl protein lacks any significant regions of homology

except for the presence of a possible protein-protein interaction-mediating C_4HC_3 zinc finger domain known as a PHD finger.

(iii) Polyhomeotic

Polyhomeotic (ph) is a large, 1589 amino acid protein that associates with polytene chromosomes at sites indistinguishable from Pc (DeCamillis et al. 1992). In fact, co-immunoprecipitation experiments have demonstrated an association between Pc and ph (Franke et al. 1992). Analysis of the amino acid sequence of ph identified a number of motifs that could mediate the protein-protein interactions of ph, including a C4 zinc finger, and a region with partial homology to a helix-loop-helix motif reviewed in (Simon 1995). Despite the association with polytene chromosomes, there is no evidence that the ph protein has the ability to bind DNA directly, similar to the other members of this family.

As with other PcG genes, partial loss of function mutations in the *ph* gene result in homeotic transformations resembling the phenotypes caused by gain of function mutations in the BX-C and ANT-C (Dura et al. 1985). However, there are several differences between mutations in *ph* and those in the defining polycomb member *Pc*. First, the hypomorphic alleles of *ph* lack detectable embryonic homeotic phenotypes and fail to show any evidence of head segment transformations (Dura et al. 1985). Second, null alleles of *ph* show pleiotropic effects ranging from cell death in the ventral epidermis to misrouting of central nervous system axons (Dura et al. 1987; Smouse et al. 1988; Smouse and Perrimon 1990). Taken together, these data suggest that ph and Pc have both overlapping and distinct functions.

(iv) Posterior sex combs

Posterior sex combs (*psc*) encodes a large protein (1603 amino acids) that has been found to immunolocalize to about 80-90 sites on polytene chromosomes (Rastelli et al. 1993). About three-quarters of these sites overlap with the sites identified for Pc and ph, suggesting that these three proteins associate with the chromosomes in a complex. In a number of embryos lacking the Psc gene product, the presence of ectopic hairs located between both the thoracic and abdominal ventral setae belts reflects the ventral to dorsal transformations of cells (Jurgens 1985; Adler et al. 1991). The *Psc* gene maps adjacent to a related gene, *Suppressor two of zeste* (*Su(z)2*). Su(z)2 contains a highly homologous (37.4% identity with Psc) 200 amino acid domain that includes a C_3HC_4 zinc finger motif called a RING finger (Brunk et al. 1991; van Lohuizen et al. 1991a). Although not classified as a polycomb gene per se, removal of a single allele of *Su(z)2* enhances the embryonic lethality of a *Psc* null allele (Adler et al. 1991). This synergy coupled with the significant homology that Su(z)2 shares with Psc strongly suggests that the two genes may share some functional redundancy.

(v) Zeste and Enhancer of zeste

The *zeste* gene product can bind *in vitro* to the regulatory regions of several *Drosophila* genes such as *white*, *Ubx*, and *antennapedia* (Benson and Pirrotta 1988). Zeste DNA binding site have been identified throughout the Ubx regulatory region, both proximal to the promoter and further upstream (Chen and Pirrotta 1993). *In vivo*, the Zeste protein can be found by immunolocalization to associate with at least 60 sites on salivary gland polytene chromosomes, including BX-C and ANT-C (Pirrotta et al. 1988). In addition to its DNA binding properties, the Zeste protein can also form homo-oligomers (Chen and Pirrotta 1993). One proposed model for Zeste action posits that the

binding of Zeste to both promoter-proximal and distal sites and the subsequent self-aggregation of the protein could bring other bound transcription factors into closer proximity, allowing for a more efficient effect on the Ubx (Biggin et al. 1988; Pirrotta 1991; Laney and Biggin 1992).

Particular alleles of *zeste* can repress the expression of the *white* gene in the fly eye. This inhibition depends on the proximity of the two copies of the *white* gene such as is produced by the pairing of homologous chromosomes (reviewed in Pirrotta 1991). Screens designed to uncover either enhancers or suppressors of this ability to repress the white gene have identified a panel of genes, including several belonging to the polycomb group. The isolation of PcG genes in these screens suggests that the mechanism of repression used by Zeste and the PcG complex may be related.

One of the PcG proteins identified in these screens was the 760 amino acid protein Enhancer of zeste [E(z)]. Like most other PcG proteins, the sequence of E(z) contains a cysteine rich region. However, unlike the other identified polycomb members, the spacing of the cysteine residues in E(z) is not consistent with any of the known zinc finger motifs (Jones and Gelbart 1993; reviewed in Simon 1995). Interestingly, E(z) possesses a C-terminal SET domain of 130 amino acids that bears significant homology (41% identity, 68% similarity) to the trithorax protein reviewed in (Francis and Kingston 2001). This SET domain is also found in a suppressor of position effect variegation, Su(var)3-9 (Tschiersch et al. 1994). Mutational studies of Su(var)3-9 have revealed that the SET domain is required for the histone methyltransferase activity of Su(var)3-9 (Rea et al. 2000; reviewed in Francis and Kingston 2001). However, the SET domain of E(z) does not seem to share this activity (Rea et al. 2000).

There are alternate hypotheses to explain the function of this conserved SET domain. Since the trxG normally antagonizes the PcG, the presence of this region of homology in E(z) has led to the speculation that it is this domain that will allow both the PcG and trxG to interact with the same target. In fact, there is evidence that E(z) can behave as either a trithorax-like activator or a polycomb-like repressor of particular Hox genes at different developmental stages and in different tissues (LaJeunesse and Shearn 1996).

A temperature sensitive allele of *E(z)* has yielded important insights into the mechanism by which PcG proteins regulate the expression of Hox gene clusters. In *E(z)* ts-mutants, at the restrictive temperature, the expression of the BX-C genes is initially normal. However, about 5-7 hours after the eggs are laid, the maintenance of the anterior boundaries of the BX-C genes begins to deteriorate and eventually leads to more widespread ectopic expression (Struhl and Brower 1982; Jones and Gelbart 1993). This deregulation occurs earlier than that seen with the other PcG family members, leading to the hypothesis that E(z) plays a different role in the repression of Hox genes (Jones and Gelbart 1993). In fact, it has recently been shown that E(z) participates in a PcG complex completely distinct from that of Pc, ph and Psc (Ng et al. 2000).

(vi) Extra sex combs

The "extra sex combs" mutation (*esc*), originally identified by Slifer in 1940's, causes ectopic Hox gene expression and this misregulation produces the additional male sex combs observed (Slifer 1942; Struhl 1981). Even though this phenotype places *esc* firmly in the PcG, other mutants have revealed a unique role for this gene in initially setting up the maintenance of Hox gene repression. A temperature sensitive allele of *esc* showed that this protein plays a critical role primarily in early embryogenesis (Struhl and

Brower 1982). *Pc* mutations, by comparison, are required for the maintenance of gene repression throughout development (Lewis 1978; Struhl 1981; Rastelli et al. 1993; Beuchle et al. 2001). Consistent with its early role in development, *esc* mRNA is observed predominantly in embryonic stages (Gutjahr et al. 1995). Furthermore, phenotypes associated with *esc* mutations seem to be solely due to ectopic expression of *Hox* genes (Struhl and Akam 1985; Simon et al. 1992). This is in sharp contrast to the other PcG members that, in addition to maintaining the repression of *Hox* genes, are involved in the regulation of gap genes (Pelegri and Lehmann 1994) and segment polarity genes such as *engrailed* (Moazed and O'Farrell 1992). These observations have led to the idea that the esc protein may play an important part in the transition between the initial repression and the maintained repression of Hox clusters in the early embryo (Gutjahr et al. 1995; Sathe and Harte 1995).

Esc is a 425 amino acid protein characterized by the presence of seven tandem WD-40 repeats (Gutjahr et al. 1995; Sathe and Harte 1995) that are believed to be used as a protein-protein interaction module. One potential hypothesis as to the function of these repeats comes from the observation that a similar domain is present in *Drosophila* TAFII80, a factor present in the general transcription factor TFIID (Albright and Tjian 2000). Gutjahr et al proposed that Esc might use these WD-40 repeats to compete with TAFII80 for binding to the basal transcriptional machinery and, in this fashion, gain access to the DNA (Gutjahr et al. 1995). Other factors could then be recruited to stabilize the repressor complex.

(vii) Pleiohomeotic

Mutant alleles of *pleiohomeotic* (*pho*), also called *lethal(4)29*, display a number of phenotypic transformations associated with the PcG, including ectopic sex combs and thoracic segment transformations (Gehring 1970,(Breen and Duncan 1986). Additionally, Hox genes of the BX-C cluster are derepressed in *pho* mutant flies. Flies bearing hypomorphic alleles of *pho* exhibit partial transformations of antennae into legs, pattern duplications and deficiencies in the legs, partial transformations of abdominal segments into more posterior segments and other transformations (Girton and Jeon 1994). Taken together, these data identify *pho* as a bona fide member of the *Drosophila* PcG.

Mutants that lack maternally deposited pho have also been generated. In these flies, the head and segmentation defects are much more severe. This early embryonic lethality, prior to the time when the PcG would be required for maintenance of BX-C repression, has led some to hypothesize that *pho* plays a critical role in other distinct events in very early embryonic development (Breen and Duncan 1986). However, the possibility exists that pho could play an important part in the earlier-acting PcG complex (see below).

PHO is a 520 amino acid protein that, by sequence homology analysis, appears to be the *Drosophila* homologue of the mammalian transcription factor Yin-Yang-1 (YY1). The DNA binding zinc finger region shows the highest degree of sequence conservation with 112/118 amino acids identity throughout the four zinc fingers and a remarkable 100% identity over fingers 2 and 3 (Brown et al. 1998). Given this amount of similarity, it is not surprising that PHO and YY1 bind the same 17 bp sequence in gel shift assays (Brown et al. 1998). One significant difference between the sequences of PHO and YY1 is the lack of a recognizable transactivation domain in PHO, leading to the speculation

that although YY1 has been implicated in both activation and repression, PHO will only participate in repression.

(viii) Additional fly PcG proteins

Many other genes have been placed into the polycomb group by virtue of phenotypic transformations associated with either loss or gain of function alleles, or association with other previously identified PcG proteins. Genes such as *Additional sex combs*, *Sex comb extra* and *Sex comb on midleg* (*Scm*) have been identified by virtue of the homeotic transformations observed upon mutation of the individual genes (Breen and Duncan 1986). The protein products of these genes share some regions of homology, consisting predominantly of zinc fingers or other domains important for protein-protein interactions, and all lack any known DNA binding motifs. Recently, dRING1, a Drosophila homologue of the mammalian PcG protein RING1, was identified and, like many of the other PcG proteins, was reported to localize with Pc to discrete foci on polytene chromosomes (Saurin et al. 2001).

Recently a new class of genes has been linked to the PcG that provides some insight into potential mechanism of polycomb-mediated repression of target genes. Deficiencies in a gene encoding *Drosophila histone deacetylase 1* (*HDAC1*) were found to enhance the ectopic sex comb transformations associated with mutations in *Pc* (Chang et al. 2001). Further analysis revealed dramatic increases in the expression levels of several Hox genes, consistent with a requirement for HDAC1 in PcG repression. In addition, HDAC1 was found to co-localize to ~70% of the polytene chromosome binding sites identified for these proteins (Chang et al. 2001). Previously, mutations in *dMi-2*, a *Drosophila* homologue of a subunit of a histone deacetylase complex with nucleosome

remodeling activity, were demonstrated to enhance the transformations associated with Pc mutations (Kehle et al. 1998). These data point to an important link between chromatin modification and silencing mediated by the PcG complex.

b) *Drosophila* Trithorax group proteins

While the maintenance of the repression initiated by the gap genes requires an intact PcG complex, the trithorax group (trxG) of proteins is necessary for the continued expression of Hox genes in regions where they have been activated by the pair-rule genes (Mahmoudi and Verrijzer 2001). There are two types of mutations that have led to the isolation of trxG genes. The first class of mutations mimic loss of function Hox gene phenotypes. *Trithorax* (*trx*), the founding member of this group, was originally identified in this fashion (Ingham 1981a; Ingham 1998). The 400 kDa protein product produced from this gene is required for development from early embryogenesis through the late larval stages (Ingham 1981b; Breen and Harte 1993). Trx contains two recognizable protein motifs, a PHD zinc finger domain and a SET domain (Kuzin et al. 1994; Sedkov et al. 1994). As mentioned previously, the 130 amino acid SET domain has been shown to be essential for the histone methyltransferase activity of Su(var)3-9, but there is no evidence that it plays the same role in trx (Rea et al. 2000).

An alternative approach for isolating trxG genes is to search for mutations that suppress Pc-induced homeotic phenotypes in a dose-dependent manner (Kennison and Tamkun 1988; reviewed in Kennison 1995). Removal of a single copy of the *brahma* (*brm*) gene prevents the homeotic transformations caused by mutations in the *Pc* gene (Kennison and Tamkun 1988; Tamkun et al. 1992). The 1638 BRM protein is the DNA dependent ATPase component of the *Drosophila* SWI/SNF chromatin-remodeling

complex. Originally identified in yeast, SWI/SNF complexes remodel nucleosome structure (reviewed in Kingston and Narlikar 1999). These alterations in nucleosome conformation can allow transcriptional activators access to their binding sites and thereby allow the trxG to maintain Hox genes in an "on" state (Francis and Kingston 2001). Consistent with this idea, two additional BRM complex components, *Osa* and *Moira*, have also been isolated as suppressors of the homeotic phenotypes produced by *Pc* mutations (Collins et al. 1999; Crosby et al. 1999; Kal et al. 2000).

As mentioned above for *E(z)*, it is possible for a particular gene to exhibit characteristics of both a PcG gene and a trxG gene under different cellular contexts. At least two other genes, *zeste* and *GAGA* or *trithorax-like*, have been identified that can behave as a member of either group as well. A possible model for Zeste action has been described above. Recently, an additional mechanism for Zeste action has been discovered. Zeste has been shown to interact with the Moira subunit of the BRM nucleosome-remodeling complex (Kal et al. 2000). At least *in vitro*, the BRM complex, when recruited by Zeste, can activate Zeste transcription (Kal et al. 2000). However, Zeste has also been purified as a member of a polycomb repressor complex. These associations among Zeste, the polycomb complex, and the BRM nucleosome remodeling complex could also be used to explain how Zeste can behave as both a PcG and trxG member. Perhaps Zeste recruits the BRM complex to promoters and depending upon the other associated proteins, either enhances the activation or the silencing of that particular locus.

GAGA was first identified as a transcriptional activator of many genes including *Ubx* (Biggin and Tjian 1988; Soeller et al. 1988; Mahmoudi and Verrijzer 2001).

Subsequently the GAGA factor was found to be encoded by the *trithorax-like* locus (Farkas et al. 1994). Trithorax-like plays a critical role in maintaining the expression of the *Ubx* and *Abd-B* genes of the BX-C (Farkas et al. 1994). GAGA, like Zeste, is a sequence specific DNA binding protein which binds as a large oligomer to multiple sites in the promoters of its target genes (Espinas et al. 1999; Katsani et al. 1999) and has been shown to be associated with a chromatin remodeling activity (Tsukiyama and Wu 1995). However, the function of GAGA *in vivo* is not well understood. In addition to its involvement in the maintenance functions of the trxG, GAGA has been shown to recruit PcG complexes (Strutt et al. 1997; Horard et al. 2000; Busturia et al. 2001). One possibility is that factors such as GAGA merely function as platforms which the trxG or PcG can assembly onto and either activate or repress target genes.

D. The Composition of *Drosophila* PcG complexes

Immunolocalization experiments have demonstrated that several of the PcG proteins co-localize to many of the same sites on polytene chromosomes, suggesting that these proteins will function together as part of a multimeric-silencing complex. Preliminary studies showed that Pc and ph were present in a complex that contained some 10-15 additional proteins (Franke et al. 1992). Using a series of assays ranging from yeast-two-hybrid to co-immunoprecipitation, Pc, ph and Psc were shown to associate with one another (Strutt and Paro 1997; Kyba and Brock 1998). Formaldehyde cross-linking and chromatin immunoprecipitation experiments demonstrated the binding of Pc, ph and Psc to identical regulatory elements in the *engrailed* promoter. These results indicate that

Drosophila PcG proteins form large complexes that can localize to regulatory sites and presumably repress transcription.

However, even amongst the Pc-ph-Psc containing complexes, there is evidence for the formation of distinct combinations. While the three PcG proteins Pc, ph and Psc co-localize to many of the sites on polytene chromosomes, some sites exist that are non-overlapping (Rastelli et al. 1993). Chromatin immunoprecipitations performed on a different gene, *invected*, showed that ph and/or Psc were not detectable at Pc binding sites (Strutt and Paro 1997). Additionally Pc was absent from the complex observed at sites in *Abd-B* (Strutt and Paro 1997). Therefore, although there is a great deal of evidence suggesting that the PcG proteins function in large multiprotein silencing complexes, the exact composition of these complexes at different sites remains to be determined.

Biochemical purification provides an alternative approach to define the components of *Drosophila* PcG complexes. For this purpose, *Drosophila* lines were generated that expressed either Flag-tagged ph or Flag-tagged Psc. Using several purification steps, including an anti-Flag antibody affinity step, embryo extracts from either ph- or Psc-transgenic flies yielded a PcG complex named the Polycomb repressive complex 1 (PRC1) (Shao et al. 1999). The PRC1 was enriched for 11 proteins including Psc, Pc, ph, and Scm (Shao et al. 1999). Recently, the PRC1 has been re-examined and found to be comprised of ph, Psc, Pc dRING1 and sub-stoichiometric amounts of SCM (Saurin et al. 2001). Again, while these data strongly suggest that the PcG proteins act together in a large complex, the notable absence of other PcG proteins, such as Pcl, implies the existence of additional Polycomb repressive complexes (Shao et al. 1999).

Further biochemical analyses have identified 30 additional proteins that can associate with the PRC1 from early *Drosophila* embryo extracts (Saurin et al. 2001). Perhaps the most surprising proteins isolated in this manner were six *Drosophila* TAFII (dTAFII) proteins. TAFII proteins were originally found through their association with the general transcription factor TFIID and serve as coactivators or selectivity factors for at least a subset of promoters (reviewed in Albright and Tjian 2000). However, in these previous cases, the TAFIIs were implicated in transcriptional activation functions. Saurin *et al* hypothesize that the dTAFII proteins present in the PRC1 provide a direct link between the PcG and the transcriptional machinery that is bound to promoter DNA (Saurin et al. 2001). In fact, the presence of both the dTAFIIs and the DNA binding protein Zeste in the PRC1 may provide clues about the manner in which the PRC1 becomes localized to the DNA.

While different permutations of the polycomb complexes described above almost certainly exist, there is at least one polycomb complex distinct from the one containing Pc-ph-Psc. Biochemical purification of the PRC1 demonstrated that the Esc and E(z) proteins did not co-fractionate with the Pc-ph-Psc-Scm complex (Shao et al. 1999). Yeast-two-hybrid and co-immunoprecipitation experiments have demonstrated a direct association between Esc and E(z) and not between these proteins and Pc, ph or Psc (Jones et al. 1998; Tie et al. 1998). These results strongly indicate the existence of two distinct polycomb complexes in *Drosophila.*

Recently, the Esc/E(z) containing polycomb complex has been isolated using a similar approach to that described for the Pc-ph-Psc containing complex. Purification of the 600 kDa Esc containing complex from transgenic embryos expressing FLAG-tagged Esc

protein, recovered E(z) protein, but not Pc or Psc (Tie et al. 2001). In addition, Drosophila RPD3, a histone deacetylase, was discovered in the purified Esc complex (Tie et al. 2001). Colocalization experiments revealed extensive overlap between RPD3 and E(z) in their binding sites on polytene chromosomes (Tie et al. 2001). Given the wealth of data linking histone deacetylation and repression of transcription, the presence of RPD3 in the Esc-containing polycomb complex provides a potential mechanism for the repression mediated by this complex. Recently, data from Chang et al 2001 showed that HDAC1 co-purified with Pc-ph-Psc, suggesting that, even though the individual players may differ, the underlying mechanism of repression is conserved between the two complexes (Chang et al. 2001).

E. Targeting of *Drosophila* PcG complexes

Despite the great deal of progress that has been made in the identification of the protein components of the PcG complex(es), much less is known about the mechanism by which these complexes associate with the DNA. In fact, in mammalian systems almost nothing is understood about the *cis*-acting sequences and the specific PcG factors that bind them. In *Drosophila*, polycomb response elements (PREs) have been isolated that confer PcG dependent silencing (Muller and Bienz 1991; Simon et al. 1993; reviewed in Sigrist and Pirrotta 1997; Satijn and Otte 1999a). PREs have been identified in many of the homeotic genes as well as *engrailed* and surprisingly, in the PcG gene *polyhomeotic* (Sigrist and Pirrotta 1997). These PREs are quite large (100-300 base pairs) and can mediate the repression of a reporter gene that has been introduced into the fly genome by a P-element transposon (Rastelli et al. 1993; Chan et al. 1994; Pirrotta et al. 1995).

However, the DNA sequences of the PREs are quite divergent. This fact, coupled with the inability of most PcG proteins to bind DNA, has led to questions about how the PREs function in PcG mediated repression.

One clue as to the mechanism of PRE function came from the observation that PcG complexes can bind to PREs when they are placed by transposon-mediated insertion into the polytene chromosomes (Zink et al. 1991; Chan et al. 1994; Zink and Paro 1995; Satijn and Otte 1999a). These data led to a model wherein the PRE would recruit the polycomb complex to DNA and nucleate the formation of PcG repressive structures on target genes (Pirrotta and Rastelli 1994; Paro 1995). In a *Drosophila* cell culture-based system, formaldehyde cross-linking and chromatin immunoprecipitation experiments demonstrated that the Pc protein was strongly associated with the PREs from inactive genes in the BX-C (Orlando and Paro 1993). This association was consistent with the nucleation model of PcG action in that the Pc protein was found over a 240 kb stretch encompassing the *Ubx* and *Abd-A* genes and their regulatory regions. In contrast, the Pc protein was not detected in association with the transcriptionally active *Abd-B* gene (Orlando and Paro 1993). The observation that flies are sensitive to PcG gene dosage further supports the nucleation model.

More recent findings have led to a refining of this model. Improved chromatin immunoprecipitation experiments have shown that the distribution of the Pc protein on the genes in the BX-C is not as broad as previously observed (Strutt et al. 1997; Strutt and Paro 1997). Instead, the Pc protein was found concentrated at a few discrete sites, many of which coincided with known PREs (Strutt and Paro 1997). Furthermore, the Pc protein only spread in a local area of a few kilobases rather than the hundreds of kb seen

in prior studies. Moreover, no differences were observed between inactive and active BX-C genes in terms of restriction enzyme accessibility (Schlossherr et al. 1994). Along these lines, removal of a PRE by recombination results in an abrogation of PcG mediated silencing (Busturia et al. 1997). These results are more consistent with a model in which PcG proteins are concentrated at particular sites and, from there, "organize" rather than "nucleate" repression of genes over long distances (reviewed in Francis and Kingston 2001).

II. Identification of mammalian PcG proteins

The *Drosophila* polycomb group is a set of genes described above that, when mutated, display similar homeotic transformation phenotypes (Satijn and Otte 1999a). Many mouse and human homologues of these *Drosophila* PcG genes have been identified. As in *Drosophila,* these mammalian proteins have been shown to play important regulatory roles in the maintenance of Hox gene repression. However, the PcG family in mammals appears more complex. There are many more PcG protein players that regulate other targets in addition to the Hox genes.

A. Mammalian Pc-ph-Psc complex

(i) Bmi1

Perhaps the best known mammalian PcG gene is *Bmi1*. *Bmi1* was originally isolated in a screen for genes that could cooperate with the *Eμ–myc* transgene in the formation of B-cell lymphomas (Haupt et al. 1991; van Lohuizen et al. 1991b). To study the connection between the c-myc transcription factor and tumorigenesis, transgenic mice

were engineered to constitutively overexpress c-myc under the control of the immunoglobulin heavy chain enhancer (Eμ) (Adams et al. 1985). These Eμ–myc mice, which express abundant levels of c-myc exclusively in the B-cell compartment, eventually die of B-cell lymphoma after a variable latency period that can be as long as 5 months (Adams et al. 1985). The delay in the onset of tumorigenesis coupled with the observation of a clear "pre-neoplastic" state led to the hypothesis that a spontaneous mutation in an independent gene must cooperate with the c-myc transgene in lymphomagenesis. To identify these genes, newborn Eμ–myc mice were infected with Moloney murine leukemia virus (Mo-MLV) and monitored for a decrease in the latency period. One of the genes, whose expression was increased in nearly half of the accelerated tumors due to the nearby proviral insertion, was called *B lymphoma Mo-MLV insertion region 1* (*Bmi1*).

The predicted 324 amino acid Bmi1 polypeptide was highly homologous to two *Drosophila* PcG proteins: Posterior Sex Combs (Psc) and Suppressor two of zeste (Su(z)2) (Brunk et al. 1991; van Lohuizen et al. 1991b). Alignments of these three proteins revealed that the highest sequence identity was located at the N-terminus in a domain encoding a putative zinc finger motif. The C_3HC_4 RING finger (Freemont et al. 1991) is most closely related to motifs present in the RAD18 *Saccharomyces cerevisiae* DNA repair protein, the V(D)J recombination protein RAG-1 and some viral DNA binding proteins (Haupt et al. 1991; van Lohuizen et al. 1991b). At the present time, however, it has not been demonstrated that the Bmi1 protein can bind to DNA directly. In fact, the zinc finger domain is required for some of the many protein-protein interactions in which Bmi1 has been found to participate. Additionally, the central

portion of the Bmi1 protein sequence contains a predicted helix-turn-helix-turn-helix-turn motif that has also been implicated in protein-protein interactions.

(ii) Mel-18

While Bmi1 is arguably the best known mammalian polycomb group protein, many other members have been identified through either homology-based or interaction-based approaches. Mel-18 is a 342 amino acid protein with 93% homology to the Bmi1 protein, including the N-terminal RING finger and the so-called H-T-H-T-H-T motifs (Ishida et al. 1993). Unlike Bmi1, however, mel-18 has been reported to bind specifically to the DNA sequence 5'-GACTNGACT-3' (Kanno et al. 1995). Furthermore, mel-18 can repress transcription when its cognate binding site is placed upstream of a reporter gene (Kanno et al. 1995). Thus, although there is a high degree of homology between mel-18 and Bmi1 in their predicted secondary structure, the degree of functional overlap between these two proteins is unclear.

(iii) polyhomeotic

The genetic yeast-two-hybrid system has also been widely utilized in the search for mammalian PcG proteins. Using the full length Bmi1 protein as bait, Gunster *et al* identified two human homologues, HPH1 and HPH2, of the *Drosophila Polyhomeotic* (*ph*) gene as Bmi1-interacting proteins (Gunster et al. 1997). The members of the mammalian family of *Polyhomeotic* genes share several regions of homology. The first two, designated as Homology Domains I and II, are involved in protein-protein interactions. In between these two domains lies a C_2-C_2 zinc finger motif whose exact function is unclear, but it is part of the sequences required for the interactions between the ph proteins and their associated partners.

(iv) Mammalian Pc family

Using the sequence from the *Drosophila* Pc chromodomain as a probe, Pearce *et al* isolated the first mouse Pc homologue, M33 (Pearce et al. 1992). This 519 amino acid protein shares significant homology with Pc both in the N-terminal chromodomain and in a 30 amino acid stretch at the C-terminus (Pearce et al. 1992). The fact that M33 is able to rescue part of the Pc mutant phenotype in transgenic flies strongly implies that it is an actual mammalian Pc homologue (Muller 1995). When fused to the Gal4 DNA binding domain, M33 is able to potently repress transcription of a reporter gene and this repression function maps to the conserved C-terminal domain (Schoorlemmer et al. 1997). Interaction studies suggest that this acidic region is important for the participation of M33 in large silencing complexes (Franke et al. 1995; Muller 1995). In light of these findings, M33 is also referred to as mPc1.

To date, there are two additional mouse and human homologues of the *Drosophila* Pc protein. In general, these proteins are highly similar to one another in both sequence and known activity. All of the members of this family are found localized to discrete nuclear foci now known as PcG bodies (Satijn et al. 1997; Schoorlemmer et al. 1997; Bardos et al. 2000). Each possesses a highly conserved N-terminal chromodomain, just like the founding member of this family M33. They all contain a highly homologous stretch of acidic amino acids, known as a "C-box" at their C-termini. This domain, as in M33, has been shown to be necessary and sufficient for protein-protein interactions (Franke et al. 1995; Muller 1995; Bardos et al. 2000; Hemenway et al. 2000). Despite its presence in all the members of this group, the C-box region also has been ascribed different functions depending upon which particular protein it resides. The ability of hPc2 to repress

transcription is relieved in mutants that lack this C-box domain (Satijn et al. 1997). However, in hPc3, this domain is dispensable for repression (Bardos et al. 2000). In conclusion, the mammalian Pc homologues identified thus far are highly similar proteins in both sequence and function. However, there are hints that these proteins may also possess some distinct functions.

(v) RING

Given the status of Bmi1 as a cooperating oncogene with c-myc, there is interest in identifying all of the proteins that are present in a Bmi1-containing polycomb complex. In addition to HPH1 and HPH2, other yeast-two-hybrid assays have shown that murine dinG/RING1B is also a Bmi1 interacting protein (Hemenway et al. 1998). In mice, there are two cDNAs, *Ring1A* and *Ring1B*, which are encoded by two different genes and are each highly homologous to the human *RING1* genes (Schoorlemmer et al. 1997). The human *RING1* gene was originally isolated in association with the human major histocompatability complex on chromosome 6 (Hanson and Trowsdale 1991). At the time, its function was completely unknown and the only recognizable motifs present in the protein were a C-terminal glycine rich region (27% glycines) and an N-terminal zinc finger related domain called a RING finger.

At about the same time, Ring1A and Ring1B were also isolated as cDNAs whose protein products could interact with the mouse Polycomb (Pc) homologue M33 in a yeast-two hybrid screen (Schoorlemmer et al. 1997). Carrying this analysis even further, both mouse and human Ring1 proteins have been shown to interact, in yeast-two-hybrid screens, with additional mouse and human Pc family members (Bardos et al. 2000; Hemenway et al. 2000). These interaction screens strongly suggested that Bmi1, Ring1

and all the mammalian PcG proteins were present in a large complex, consisting of many protein-protein interactions.

(vi) RYBP

Finally, in a yeast-two-hybrid screen aimed at the identification of Ring1A interacting proteins, a protein called Ring1 and YY1 binding protein (RYBP) was isolated (Garcia et al. 1999). This 228 amino acid protein has an N-terminal C_2C_2 zinc finger motif, a high content of basic residues (41/228) and a C-terminus characterized by the overabundance of serine and threonine residues (31/84) (Garcia et al. 1999). RYBP has significant homology to a protein, YY1 associated factor (YAF2), that was identified in a yeast-two-hybrid screen using YY1 as the bait (Kalenik et al. 1997). Unlike other mammalian PcG proteins, RYBP is distributed throughout the nucleus. However, lower expression levels of RYBP revealed that the protein is capable of adopting the characteristic speckled appearance of other PcG proteins. In addition, Gal4-DNA-binding domain fusions with RYBP are able to repress transcription of a heterologous reporter gene. Taken together, these data strongly indicate that RYBP is a member of the mammalian polycomb complex.

One unusual feature of RYBP is its ability to interact with both Ring1A and YY1. Whereas both of these proteins have been implicated in polycomb function, they are believed to belong to distinct complexes *in vivo*. Whether this property of RYBP is indicative of an association between the two polycomb complexes or if they merely contain some common factors is still under investigation.

B. Mammalian Esc/E(z) complex

(i) Embryonic ectoderm development

Positional cloning of the classical mouse gastrulation mutant *embryonic ectoderm development* (*eed*) yielded a gene whose protein product has significant homology (55% identity, 74% similarity) to the *Drosophila* Esc protein (Shumacher et al. 1996; van Lohuizen et al. 1998). Like its fly counterpart, the 535 amino acid EED protein contains a WD-40 repeat domain that is believed to be important for protein-protein interactions (Sewalt et al. 1998; van Lohuizen et al. 1998). When fused to a heterologous DNA binding domain, EED, like esc, can repress transcription (Denisenko et al. 1998; Sewalt et al. 1998). RT-PCR experiments performed at early murine developmental stages showed very high levels of *eed* transcripts beginning at E5.5 and continuing through to E9.5 (Shumacher et al. 1996). These expression patterns are consistent with a role for EED in establishing an early maintenance of repression. Similarly, ESC has been predicted to play a critical part in the transition from early repression by gap genes to the subsequent maintenance of repression by the PcG. Mice mutant for *eed* confirm that this gene is, in fact, essential for early mouse development and pattern formation.

(ii) Enx1/Ezh1 and Enx2/Ezh2

Mammalian *Enhancer of zeste* homologues (*Enx1/Ezh1* and *Enx2/Ezh2*) have been identified through a combination of homology-based searches and yeast-two-hybrid screens (Hobert et al. 1996a; Laible et al. 1997). The primary difference between Enx1/Ezh1 and Enx2/Ezh2 is in their expression patterns, with Enx1/Ezh1 being primarily found in adult tissues and Enx2/Ezh2 expressed at high levels during embryonic development (Hobert et al. 1996b; Laible et al. 1997). Enx1 was isolated as

an interacting protein for the proto-oncogene Vav in hematopoietic cells (Hobert et al. 1996a). Even though the authors hypothesize a role for Enx1 in signal-dependent T cell activation, the biological significance of the Enx1/Vav interaction is still not known.

Alignment of the sequences from *Drosophila E(z)* and *Ezh1/Ezh2* revealed four regions of strong homology: Domain I, Domain II, a cysteine rich region and a C-terminal SET domain (Hobert et al. 1996b; Laible et al. 1997). The first two domains are important for the interaction with Vav and are therefore believed to be important for protein-protein interactions. The functions of the cysteine rich region and the SET domain are unknown, but SET domains have been implicated in histone modifications (Rea et al. 2000) and see above. Additionally, Ezh2 enhances position effect variegation phenotypes when expressed in flies (Laible et al. 1997). Taken together, these results indicate that there are two human homologues of *Drosophila* E(z) and they are likely to play similar roles in the PcG complex.

C. Mammalian trithorax complex

The human *mixed lineage leukemia* (*mll*) gene was originally identified as a common target of chromosomal translocations in human acute leukemias (Gu et al 1992; Tkachuk et al. 1992). MLL and its murine homologue are strikingly similar to the *Drosophila* trithorax protein especially in the C-terminal SET domain (81% similarity) and the centrally located zinc PHD fingers (reviewed in Gould 1997). However, unlike its *Drosophila* counterpart, the mammalian MLL protein possesses three small domains with significant homology to the AT hook motifs present in the HMG-I(Y) protein. High mobility group (HMG) proteins are chromatin-associated factors that have been proposed

to play many roles in transcriptional regulation (Reeves and Nissen 1990). The presence of this domain in MLL has led to speculation that it is the AT hook motif that allows MLL to bind to DNA (Tkachuk et al. 1992).

Disruption of the *mll* gene in mice leads to a profound loss of Hox gene expression and embryonic lethality (Yu et al. 1995). Consistent with the studies in *Drosophila*, *mll* homozygotes do initiate Hox gene expression at around E8.5, but fail to maintain expression, resulting in the complete absence of some Hox transcripts by E10.5 (Yu et al. 1998). *Mll* heterozygous mutant mice also exhibit gross abnormalities. These mice are growth retarded, display both anterior and posterior homeotic transformations of the axial skeleton, and have some hematopoietic deficiencies (Yu et al. 1995). Thus, the *mll* gene is part of a conserved regulatory trxG network that is necessary for maintaining the expression patterns of at least the homeotic genes. At present, however, the molecular mechanisms for this maintenance are unclear.

D. Composition of the mammalian PcG complexes

Both biochemical and genetic studies in *Drosophila* point to the existence of at least two distinct polycomb complexes. Mutant mouse models combined with protein interaction studies strongly suggest that an analogous situation exists for the mammalian polycomb complex. Although there may be further protein permutations possible within each subset of PcG complexes, it appears clear that there is at least an "early" and a "late" mammalian polycomb complex.

The "early" PcG complex is defined by the time of onset of the phenotypes in mutant mice and "guilt by association". Yeast-two-hybrid assays have identified Enx1 and Enx2

as specific interacting proteins for EED (Sewalt et al. 1998; van Lohuizen et al. 1998). In these experiments, no interactions could be found between EED and the proteins in the Bmi1-containing "late" PcG complex. Co-immunoprecipitation experiments and immunostaining of both transfected and endogenous proteins confirmed that EED and ENX can associate with each other, but not with any of the members of the Bmi1 containing PcG complex tested (Sewalt et al. 1998; van Lohuizen et al. 1998).

In mammalian cells, the "early" polycomb complex may also differ in its mode of repression. Again, similar to the Esc complex in the *Drosophila*, EED has been shown to be capable of associating with histone deacetylase enzymes (HDAC) (van der Vlag and Otte 1999; Tie et al. 2001). In mammalian cells, this association appears to be specific for EED as several members of the Bmi1-containing PcG complex failed to interact with the HDAC enzymes. Additionally, inhibition of HDAC activity with trichostatin A abrogated the ability of EED to inhibit the transactivation of a reporter construct, despite leaving the repressive capabilities of the other PcG complex intact (van der Vlag and Otte 1999). Taken together, these data demonstrate the existence of at least two alternative polycomb complexes that repress their target at different times through distinct mechanisms.

The "late-acting" mammalian polycomb complex has been defined in similar terms. Protein interaction experiments such as the yeast-two-hybrid assay have been used extensively in defining this PcG complex. Ring1, for example, has been shown to interact with Bmi1, hPc2, and itself all through distinct regions (Satijn and Otte 1999b). Using a similar approach, Bmi1 was found to be capable of interacting with Ring1, hPc2, HPH1, HPH2 and itself (reviewed in Satijn and Otte 1999a). The domains responsible

for some of these Bmi1 interactions were overlapping, however, suggesting that all of these proteins may not be present in the same complex. In addition to yeast-two-hybrid analysis, the proteins of this "late" PcG complex all co-localize in large nuclear speckles. While the biological significance of this localization is not clear, it has been used as evidence for these proteins associating in a large PcG complex.

E. Targeting of mammalian PcG action

Despite the progress made in understanding the mechanism, by which *Drosophila* PREs can facilitate assembly of PcG complexes and subsequent repression, very little is known about how vertebrate PcG proteins interact with DNA. In fact, the DNA sequences/DNA binding factors responsible for targeting the mammalian polycomb complex are largely unknown (Satijn and Otte 1999a). Even with this lack of information, some clues have emerged that heterochromatin and histone modifications may play an important role in PcG targeting.

In human cell lines, at least three polycomb proteins, RING1A, Bmi1 and hPc2 co-localize in nuclear structures termed PcG bodies (Saurin et al. 1998). Using a combination of protein localization and fluorescent in situ hybridization (FISH) techniques, Saurin *et al* further demonstrated that these PcG bodies preferentially associate with heterochromatic regions (Saurin et al. 1998). Fractionation studies have confirmed that Bmi1 is specifically retained with chromatin associated nuclear proteins (Voncken et al. 1999). Although these studies fail to yield a specific DNA binding site for the PcG complex, they do suggest that these complexes are associated with DNA and that the formation of heterochromatin may play a role in PcG mediated repression.

The chromatin organization modifier (chromodomain) is a highly conserved stretch of about 50 amino acids originally identified as a region of significant homology between *Drosophila* Pc and heterochromatin protein 1 (HP1) (Paro and Hogness 1991). Since then, at least 40 chromodomain-containing proteins have been isolated, including three mammalian homologues of HP1 (reviewed in Jones et al. 2000). In addition to the N-terminal chromodomain, the mammalian HP1 proteins also possess a C-terminal repeat of the chromodomain called a chromoshadow domain (Aasland and Stewart 1995). These bipartite proteins are believed to function as adaptors in recruiting multiprotein complexes to heterochromatin (Aasland and Stewart 1995).

Recent studies have revealed a potential mechanism for how chromodomains function. Methylation of lysine 9 in the tail of histone H3 was found to generate a binding site for the chromodomains of mammalian HP1 proteins and the murine Pc protein, mPc1 (Bannister et al. 2001; Lachner et al. 2001). The subsequent model proposed that methyltransferases such as SUV39H1 could be recruited to DNA and methylate histone proteins, creating binding sites for HP1 or mPc1. Once sitting on the DNA, these proteins could utilize other protein-protein interaction domains to recruit transcriptional repression complexes. The presence of a "methyl marker" also provides a molecular mechanism to explain the stable inheritance of heterochromatic states through DNA replication (Bannister et al. 2001).

F. PcG mouse models

Vertebrate homologues for many of the *Drosophila* PcG genes have been identified. Although there may be differences in the specific mechanisms by which mammalian and

fly PcG complexes participate in silencing, knockout mouse phenotypes strongly suggest that at least some of their functions are conserved. In addition, mouse models for PcG family members have revealed both distinct and overlapping phenotypes, suggesting that the manner in which mammalian PcG complexes regulate target genes is anything but simple. Finally, the fact that there are two categories of PcG mutant mouse phenotypes (one early and one late) underscores the idea that there are at least two distinct mammalian PcG complexes.

(i) Genes from the embryonic ectoderm development (eed)-containing PcG complex

The *eed* mutation was originally generated as part of a series of deficiencies used to study mammalian development (Niswander et al. 1988). Mice homozygous for deletions of this gene displayed dramatic defects in gastrulation (Niswander et al. 1988; Faust et al. 1995). Subsequently, the *eed* gene was cloned and shown to be homologous to the *Drosophila* PcG gene *esc*. A re-examination of the *eed* phenotype revealed that anterior/posterior patterning of the early embryo was disrupted (Shumacher et al. 1996). This patterning normally occurs prior to the action of Hox genes, suggesting the existence of at least one other target gene for eed. However, mice carrying a hypomorphic allele of *eed* do exhibit posterior transformations of the axial skeleton, placing eed firmly in the PcG complex (Shumacher et al. 1996). Therefore, the phenotypes of *eed* mutant mice are consistent with this gene playing a key role in the function of the early murine PcG complex.

The multifunctional transcription factor YY1 has also been implicated in the "early" mammalian PcG complex. Disruption of the murine *YY1* gene leads to early embryonic lethality. *YY1*$^{-/-}$ embryos implant into the uterine wall, but fail to form egg cylinders and

are resorbed by the mother (Donohoe et al. 1999). This early lethality is characteristic of Ezh2 mutant mice as well. However, besides the requirement in early development, no other phenotype associated with the YY1 mutant mice is reminiscent of PcG genes. Heterozygous mutant *YY1* mice are growth retarded with a high incidence of exencephaly, but no segmental transformations (Donohoe et al. 1999). Therefore, whether YY1 is a bona fide PcG member is still an open question.

One of the mammalian homologues of E(z), Enhancer of zeste 2 (Ezh2), is the only mammalian PcG gene to be expressed at preimplantion stages of mouse development. YY1 is expressed this early in development, but its link to the polycomb complex is still being debated. Even though eed interacts with Ezh2, eed expression is not detectable until E5.5. Consistent with the early expression patterns, a significant proportion of mice deficient for Ezh2 exhibit very early embryonic lethality with many animals dying around the pre- to postimplantation transition (O'Carroll et al. 2001). Those mice that survive past implantation die around E8.5 with defects in cell migration during gastrulation (O'Carroll et al. 2001). This phenotype is very reminiscent of eed mutant mice that die between E7.5 and E8.5 with abnormal gastrulation (Niswander et al. 1988; Faust et al. 1995).

The *Ezh2*$^{-/-}$ mice revealed a potential role for this protein in the regulation of cellular proliferation. The category of embryos that failed to survive past implantation exhibits severe growth retardation (O'Carroll et al. 2001). Since Ezh2 is widely expressed at this time of elevated cellular proliferation, the authors hypothesize that Ezh2 might play a general role in the control of proliferation. Consistent with this hypothesis, *Ezh2*$^{-/-}$ embryonic stem (ES) cells could not be isolated due to a failure to proliferate in culture.

Therefore, Ezh2, together with its partner eed and potentially also YY1, is critical for the regulation of events during early embryonic development.

(ii) Genes from the bmi1 containing PcG complex

The defining phenotype for polycomb group mutations in *Drosophila* is homeotic transformations. Consistent with the relationship between *Drosophila* and mammalian PcG proteins, mice deficient for the PcG genes *Bmi1*, *Mph1/rae28*, *M33*, *mel-18*, and *Ring1A* all display homeotic transformations of the axial skeleton and corresponding shifts in the expression patterns of certain Hox genes (van der Lugt et al. 1994; van der Lugt et al. 1996; Takihara et al. 1997) (Akasaka et al. 1996; Core et al. 1997; del Mar Lorente et al. 2000). In fact, overexpression of Bmi1 in a transgenic mouse model also led to defects in axial skeleton formation and shifts in Hox gene expression (Alkema et al. 1995). As predicted, these changes are in the opposite direction, namely anterior instead of posterior, from those observed in the Bmi1 knockout mouse.

In addition to possessing axial skeleton transformations, mice deficient for one of the polycomb genes generally exhibit hematopoietic abnormalities. For example, *Bmi1*, *mel-18* and *Mph1/rae28* deficient mice all have severe reductions in the number of mature B cells (van der Lugt et al. 1994; Akasaka et al. 1997; Tokimasa et al. 2001). In contrast, mice overexpressing Bmi1 exhibit hyperproliferation of B and T cells leading to a high incidence of lymphoma (Alkema et al. 1997b). Further examination of B cell development in these mice using more *in vitro* systems revealed that immature B-lymphocytes consistently fail to differentiate appropriately when treated with the cytokine interleukin-7 (IL-7). These data suggest that the mammalian PcG genes participate in the regulation of cellular differentiation.

Despite the similarities in homeotic transformations and hematopoietic abnormalities among the different polycomb gene knockout mice, there are many significant differences as well. Bmi1 deficient mice exhibit a lack of coordination that manifests itself by 2-4 weeks of age (van der Lugt et al. 1994). This ataxia is believed to be due to a significant loss of neurons from the cerebellum. The molecular basis for this loss is still not completely understood, but, among the PcG members, it seems to be specific to the Bmi1 knockout mouse.

Other mammalian PcG gene knockout mice display unique phenotypes as well. The Mph1/rae28 deficient mouse dies perinatally with a host of neural crest related defects including cleft palate, parathyroid hypoplasia and cardiac anomalies (Takihara et al. 1997). Mice lacking the *mel-18* gene die between 3 and 6 weeks after birth, possibly due to the presence of obstructions in their lower intestine (Akasaka et al. 1996). This death is believed to be linked to the strong hypertrophy of smooth muscles these mice exhibit. These differences in phenotypes suggest that while the polycomb proteins may have many overlapping functions, at least in some tissues, these genes appear to have distinct functions as well.

Genetic studies in mice have demonstrated that, as in *Drosophila*, reduction in the dosage of a PcG in the context of a deficiency for another PcG gene can enhance the resulting phenotype. Two such crosses have been performed: *Bmi1*$^{-/-}$ mice with *M33*$^{-/-}$ mice and *Bmi1*$^{-/-}$ mice with *mel-18*$^{-/-}$mice. In each case the double mutant mouse exhibits more severe skeletal transformations than either single mutant mouse. These enhanced transformations are accompanied by more profound shifts in Hox gene expression (Bel et al. 1998; Akasaka et al. 2001). Synergistic interactions also produced additional skeletal

defects not observed at all in the single mutants. Taken together, these mouse crosses demonstrate that the mammalian PcG proteins act in concert to regulate Hox gene expression.

Interestingly, the PcG knockout mice also display significant differences even with regard to some of the apparently similar phenotypes listed above. Most notably, despite the fact that all the mice display some ectopic expression of Hox genes, the particular Hox genes affected differ from one knockout mouse to another. For instance, whereas the expression of *HoxA5* and *HoxC8* is shifted in *Bmi1*$^{-/-}$, *mel-18*$^{-/-}$ and *M33*$^{-/-}$ mice, *HoxC6* and *HoxC5* are only misregulated in the *Bmi1*$^{-/-}$ mouse while *HoxA7* and *HoxD4* are affected in the *mel-18*$^{-/-}$ mouse (van der Lugt et al. 1994; Akasaka et al. 1996; Akasaka et al. 2001). Additionally, even though both Mel-18 and Bmi1 play a role in apoptosis, the downstream targets involved are quite different. Although Bmi1 deficient mice show an upregulation of p19^{ARF}, thymocytes from *mel-18*$^{-/-}$ mice have normal levels of p19^{ARF} and an increase in the proapoptotic molecule Bad (Jacobs et al. 1999a; Akasaka et al. 2001). Doubly deficient mice show a synergy in apoptosis, presumably through the activation of both pathways, leading to an early lethality (E9.5) (Akasaka et al. 2001). Finally, even though the *Ring1A*$^{-/-}$ mice exhibit axial skeletal abnormalities, these are anterior transformations while the other PcG mutant mice display posterior transformations (del Mar Lorente et al. 2000).

Thus, although PcG mutant mice demonstrate clear overlapping roles for some of these genes in the control of Hox gene expression and axial skeleton formation, the exact composition of the different complexes involved is still not clear. Furthermore, even

though some of the phenotypes may be similar, PcG mutant mice are revealing that the downstream target genes involved need not be the same.

G. The PcG, cell cycle and tumorigenesis

The oncogene *Bmi1* was originally isolated by virtue of its ability to cooperate with c-myc and accelerate the induction of B-cell lymphomas (Haupt et al. 1991; van Lohuizen et al. 1991b). Subsequently Bmi1 alone has been shown to transform Rat1a fibroblasts with a similar efficiency to c-myc. Moreover, c-myc and Bmi1 synergize in the transformation of rat embryo cells (Cohen et al. 1996). Deletion analysis has determined that the N-terminal RING finger is required for transformation, but the helix-turn-helix-turn-helix motif, which is necessary for Bmi1 mediated repression, is dispensable (Bunker and Kingston 1994; Cohen et al. 1996). These cell-based results agree with transgenic mouse data in which expression of an Eμ-Bmi1 transgene lacking the RING finger fails to render the mice susceptible to lymphomas (Alkema et al. 1997b). This result is somewhat surprising since deletion of the RING finger domain, in addition to not affecting Bmi1-mediated repression, does not alter the ability of Bmi1 to interact with murine ph1 (mph1) or the ability of a Bmi1 transgene to induce anterior transformation of the axial skeleton (Alkema et al. 1997a; Alkema et al. 1997b). These studies further confirm the status of Bmi1 as an oncogene and show that its oncogenic potential could be separated from its ability to repress transcription both *in vitro* (reporter genes) and *in vivo* (Hox genes).

To more fully understand the role of Bmi1 as an oncogene and to investigate a potential mechanism for the synergy between c-myc and Bmi1, Jacobs *et al* examined the

properties of mouse embryonic fibroblasts (MEFs) prepared from *Bmi1*$^{-/-}$ embryos. These cells display all the hallmarks of premature cellular senescence: cytoplasmic enlargement, flattening of the cells, unresponsiveness to growth factors and expression of senescence associated B-galactosidase in the majority of the cells (Jacobs et al. 1999a). Additionally, when *Bmi1*$^{-/-}$ MEFs are subjected to a standard 3T3 protocol (Todaro et al. 1965), they are reported to cease proliferating after passage 3. These data strongly implicate Bmi1 in the control of cellular proliferation.

The *ink4* locus, which encodes for two tumor suppressor proteins, p16^{ink4a} and p19^{ARF}, has been shown to play a key role in cellular senescence (Alcorta et al. 1996; Hara et al. 1996; Kamijo et al. 1997). Given the premature senescence phenotype of the *Bmi1*$^{-/-}$ MEFs, Jacobs *et al* examined the levels of p16^{ink4a} and p19^{ARF} in these cells. In the absence of Bmi1, p16^{ink4a} transcripts are upregulated an average of eightfold and p19^{ARF} steady-state transcript levels increased two to threefold (Jacobs et al. 1999a). Consistent with these observations, overexpression of retroviral Bmi1 in MEFs results in the downregulation of both p16^{ink4a} and p19^{ARF}. In fact, these Bmi1 overexpressing cells could now be transformed by the addition of activated ras alone.

To extend these observations to a more *in vivo* setting, *Bmi1*$^{-/-}$*;ink4a*$^{-/-}$ doubly deficient mice were generated. As discussed above, the Bmi1 deficient mice have cerebellar defects that are believed to cause the severe ataxia these mice exhibit at 2-4 weeks of age (van der Lugt et al. 1994). *Bmi1*$^{-/-}$*;ink4a*$^{-/-}$ mice demonstrated normal behavior at 5 weeks of age and a corresponding dramatic rescue of the cerebellum phenotype (Jacobs et al. 1999a). However, although the defects in the granule cell layer of the cerebellum were almost completely rescued, other phenotypes in the brain still persisted. This partial

rescue resulted in a delayed onset and slower progression of the neurological phenotype, indicating that the *ink4* locus is only one critical target for Bmi1 *in vivo*.

The *Bmi1*$^{-/-}$ mice also display a severe hematopoeitic deficiency, having less than 5% of the number of thymocytes and splenocytes present in wild type mice. In the double knockout, *Bmi1*$^{-/-}$;*ink4a*$^{-/-}$, mice, thymocyte and splenocyte numbers are restored to up to 70% that of their wild-type littermates (Jacobs et al. 1999a). Thus, *ink4a* is a downstream target of Bmi1 function in lymphoid cells as well.

As pointed out above, Bmi1 was identified in a proviral screen for genes that cooperated with c-myc in lymphomagenesis. Removal of one copy of Bmi1, in the presence of the *Eμ–myc* transgene, significantly extended the survival of the transgenic mice by suppressing tumorigenesis (Jacobs et al. 1999b). The reason behind this lifespan extension was found to be an increase in susceptibility of immature B cells to myc-induced apoptosis. In the presence of a reduced dosage of Bmi1, p19^{ARF} levels are increased. Higher amounts of p19^{ARF} protein in these cells should activate p53 and lead to p53-dependent apoptotic response. Taken together, these data lead to a model where myc can activate either a proliferative response or apoptosis through p19^{ARF}. In the presence of high levels of Bmi1, the p19^{ARF} gene is silenced and the proliferative response of myc is favored. With lower amounts of Bmi1, the p19^{ARF} gene can now be activated, leading to an apoptotic response, probably mediated through p53.

Even though the importance of Bmi1 in murine tumorigenesis is clear, its role in human cancers is less well understood. The chromosomal region to which human Bmi1 has been mapped (10p13) is involved in chromosomal abnormalities associated with infant leukemias and some T cell lymphomas. However, the absence of specific

alterations in the Bmi1 gene leaves its precise role in tumorigenesis unclear (Berger et al. 1988; Alkema et al. 1993; Bea et al. 1999). Recently, the *Bmi1* gene was found to be amplified in a subset of mantle cell lymphomas and the expression of Bmi1 was observed to be increased in several other tumors as well (Bea et al. 2001). These new data suggest that although Bmi1 alterations in human cancers are somewhat rare, they can occur in a small, specific subset of cases. The contribution that these changes in expression make to the disease condition is currently not known.

Other polycomb group proteins have been implicated in tumorigenesis and cell cycle control. MEFs and splenocytes from *M33*$^{-/-}$ mice display significantly impaired proliferation (Core et al. 1997), suggesting that the M33 gene product normally promotes cell growth. NIH3T3 cells engineered to overexpress antisense mel-18 form tumors when injected into nude mice (Kanno et al. 1995), leading the authors to conclude that unlike Bmi1, mel-18 is a tumor suppressor. B cells from mice lacking mel-18 incorporate higher levels of [^{3}H]thymidine even in the absence of any stimulation (Tetsu et al. 1998). To extend these studies, mel-18 transgenic mice were generated. Splenic B-lymphocytes isolated from these mice were severely impaired in their ability to proliferate in response to anti-IgM stimulation (Tetsu et al. 1998). This G1 arrest was characterized by a severe reduction in c-myc expression, while other early transcriptional events, such as c-fos induction, were normal. The proliferative response in B cells from mice doubly transgenic for mel-18 and c-myc was rescued, confirming that c-myc is in fact a critical downstream target of mel-18 (Tetsu et al. 1998). These results demonstrate that while Bmi-1 and mel-18 are very similar proteins with many overlapping roles in development, they have opposing roles in the context of cellular proliferation control.

Two other mammalian PcG genes have been linked to tumorigenesis. The first is a human homologue of the Polycomb gene, *hPc2*. Expression of a deletion mutant of the hPc2 protein that is unable to repress transcription caused phenotypic changes characteristic of cellular transformation (Satijn et al. 1997). These changes were accompanied by an increase in *c-myc* expression, providing a potential explanation for their altered morphology. Expression of this putative dominant negative construct in Rat1a cells led to anchorage independent growth, a hallmark of transformation. These data strongly suggest that hPc2 can behave as a tumor suppressor gene by influencing the expression of a well-known proto-oncogene.

Finally, RING1 has also been shown to play a role in tumorigenesis. Stable overexpression of RING1 in Rat1a cells resulted in an upregulation of the oncogenes c-fos and c-jun, with a concomitant induction of anchorage independent growth (Satijn and Otte 1999b). Injection of nude mice with NIH3T3 cells engineered to overexpress RING1 resulted in the formation of tumors in 5/8 mice examined (Satijn and Otte 1999b). No tumors were found in mice injected with control NIH3T3 cells. Significantly, the tumors in these mice were observed throughout the body, mainly in the liver, but also in the kidney and intestine. These results provide strong evidence for RING1 as a putative oncogene and perhaps even a regulator of metastatic potential.

This chapter has described two proteins families involved in transcriptional regulation. The E2F proteins are critical regulators of target genes important for cell cycle progression and terminal differentiation. The polycomb group of proteins was originally identified in *Drosophila* as a set of factors essential for the maintenance of homeotic gene repression. While these proteins also possess this function in mammalian

cells, it seems apparent that they are important for the repression of additional target genes as well. In this study, I have identified a novel E2F, E2F6, which has properties consistent with being an E2F family member (Chapter 2), but also interacts with proteins from the Bmi1-containing polycomb complex (Chapter 3).

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Chapter 2

E2F6, a member of the E2F family that can behave as a transcriptional repressor

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Author's contribution: Figures 1, 2, 3, and 4

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Abstract

The E2F family of proteins is required to establish the correct cell-cycle-dependent transcription of genes that direct the process of cell division. All previously identified E2F proteins can act in a similar manner; depending on whether or not they are associated with the cell cycle inhibitors the retinoblastoma protein (pRB), p107, or p130, they can either repress or activate the transcription of E2F-responsive genes. We now report the cloning and characterization of another E2F family member, E2F6, whose structure is reminiscent of the dominant inhibitors of other transcription factor families. The dimerization and DNA binding properties of E2F6 are similar to those of the other E2F family members. However, it is not regulated by pRB, p107, or p130, and it is unable to activate transcription. Instead, it can act to repress the transcription of E2F responsive genes by countering the activity of the other E2F complexes via a pRB-, p107-, or p130-independent mechanism.

Introduction

The retinoblastoma gene (*RB1*) is one of the best studied of the tumor suppressor genes. Although it was originally identified by virtue of its absence in retinoblastomas, subsequent studies have shown that it is absent or mutated in at least one-third of all human tumors (Weinberg 1992). The product of this gene [the retinoblastoma protein (pRB)] is also an essential target of the transforming proteins of the small DNA tumor viruses (Dyson et al. 1992). In untransformed cells, the growth-suppressive properties of pRB are regulated by its cell-cycle-dependent phosphorylation (Bartek et al. 1996). This phosphorylation is catalyzed by the cell-cycle-dependent kinase cyclin D cdk4/6 (Bartek et al. 1996). Consistent with this hypothesis, many human tumors contain activating mutations within either the cyclin D1 or cdk4 genes or have lost the cdk4/6-specific inhibitor p16 (Pollock et al. 1996).

The growth-suppressive properties of pRB are dependent upon its ability to regulate a cellular transcription factor, E2F (Nevins 1992; Bartek et al. 1996). Many E2F-responsive genes have been identified, and their products are required for entry into, or passage through, the cell cycle. Consistent with its antiproliferative role, the pRB protein inhibits the transcriptional activity of E2F (Hiebert et al. 1992). Moreover, the resultant pRB/E2F complex actively represses the transcription of E2F-responsive genes by blocking the activity of adjacent transcription factors (Weintraub et al. 1992; Bremner et al. 1995; Weintraub et al. 1995). Phosphorylation of the pRB protein causes it to dissociate from E2F, thereby switching E2F-responsive genes from the repressed to the induced state.

E2F is regulated by two additional proteins called p107 and p130 (Beijersbergen and Bernards 1996). These proteins share significant sequence similarity with pRB (Dyson et al. 1992) (Beijersbergen and Bernards 1996), and in overexpression assays they repress E2F in a similar manner to pRB (Zhu et al. 1993; Starostik et al. 1996). However, genetic studies indicate that pRB, p107, and p130 have distinct properties *in vivo*. Whereas pRB is mutated in 30% of all human tumors, neither p107 nor p130 is a tumor suppressor (Weinberg 1996). Moreover, analysis of mutant mouse strains indicates that pRB is essential for development, whereas loss of p107 or p130 does not alter viability or tumor incidence (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992; Cobrinik et al. 1996; Lee et al. 1996). It is unclear exactly how the different biological properties of these proteins relate to the manner in which they regulate E2F, but pRB, p107, and p130 are known to bind to E2F at defined but distinct stages of the cell cycle (Beijersbergen and Bernards 1996).

To date, at least seven human genes have been identified that encode components of the E2F transcriptional activity (Beijersbergen and Bernards 1996). These can be divided into two distinct groups, named E2F (E2F1 through 5) and DP (DP1 and 2). The products of these groups heterodimerize to give rise to high-affinity DNA binding activity and transcriptional activation (Bandara et al. 1993; Helin et al. 1993; Krek et al. 1993; Wu et al. 1995). *In vivo* studies confirm that the endogenous E2F activity is generated from the combined properties of multiple E2F/DP complexes (Wu et al. 1995; Moberg et al. 1996). The individual E2F/DP species have different pRB, p107 and p130 binding properties. Although the DP subunit is essential, the E2F moiety mediates the specificity of this interaction. Complexes containing E2F1, 2, or 3 associate with pRB,

but not p107 or p130 *in vivo* (Dyson et al. 1993; Lees et al. 1993). In contrast, E2F4 and 5 complexes are capable of binding p107 and p130 (Beijersbergen et al. 1994; Ginsberg et al. 1994; Hijmans et al. 1995). Consistent with these findings, sequence comparisons suggest that the family of E2F proteins can be subdivided into two distinct subgroups. The pRB-specific E2Fs (E2F1 through 3) have an extended N-terminal domain that is absent in both E2F4 and 5. There is also considerable variation in the sequence of the DNA binding, dimerization, and transactivation domains between members of the two E2F subgroups (E2F1 through 3 versus E2F4 and 5). These observations have led to the hypothesis that these two subgroups will play distinct roles in vivo that will at least partially account for the different biological consequences of loss of pRB, p107, or p130.

To examine the biochemical and functional properties of the endogenous E2Fs, we had previously developed specific antisera for each of the components of the E2F family (Moberg et al. 1996). With these reagents, we have been able to demonstrate that the known E2F proteins are unable to account for all of the endogenous E2F/DP DNA binding activity (Moberg et al. 1996). In this study, we describe the cloning and characterization of an additional E2F family member, E2F6. The DNA binding and dimerization domains of E2F6 are highly related to the corresponding domains of the previously identified family members, but this protein lacks the sequences necessary for either transactivation or pRB, p107, or p130 binding. We conclude that the E2F family contains a third subgroup of proteins whose structure is highly reminiscent of the dominant inhibitors of other transcription factor families.

Materials and Methods

cDNA Identification and Characterization. GenBank, EMBL, and DDBJ databases were searched with the protein sequence QKRRIYDITNVLEG by using the TBLASTN program. The identified E2F6 human and mouse expressed sequence tags (ESTs) were obtained from Research Genetics (Huntsville, AL). A human fetal brain cDNA library (Stratagene) was screened with a 1.6-kb *Eco*RI fragment of a human EST labeled with [α-^{32}P]dCTP by random priming. Hybridization was performed at 42°C in 5X SSC/5X Denhardt's solution/30% formamide/0.5% SDS/dextran sulfate (50 μg/ml)/salmon sperm DNA (150 μg/ml). Filters were washed at 55°C for three 20-min periods in 1X SSC/0.1% SDS. Positive clones were identified by autoradiography. Exonuclease III (New England Biolabs) digestion was used to generate nested deletions of both EST and cDNA clones, which were then sequenced with Sequenase 2.0 (United States Biochemical).

Plasmid Construction. The human E2F6 ORF was amplified by PCR with the primers 6.6 (GTTAGGATCCATGCGGCACGAGAAGTTACCCAG) and 6.10 (CTCAGGATCCATCAGTTGCTTACTTCAAG) and Vent polymerase. The PCR product was digested with *Bam*HI and subcloned into pHACMV-neo-Bam to generate pCMV-HA-E2F6. The plasmids pE2F$_4$-CAT, pRSV-luciferase, pCMV-E2F1, pCMV-E2F4, pCMV-DP1, pCMV-HA-DP1, pCMV-HA-DP2, pCMV-RB and pCMV-p107 have been described (Helin et al. 1993; Wu et al. 1995; Moberg et al. 1996). The 6X His-tagged E2F6 vector was constructed by amplifying the E2F6 ORF with primers 6.6 and

6.12 (CACTAAGCTTATCAGTTGCTTACTTCAAGCA). The PCR product was digested with *Bam*HI and *Hind*III and subcloned into pQE30 (Qiagen, Chatsworth CA).

Northern Blot Analysis. The cell-line northern blot [containing poly(A)$^+$ RNA from 293 (adenocarcinoma), HeLa (cervical carcinoma), ML-1 (myeloid leukemia), T98G (neuroblastoma), MCF7 (human breast cancer cell line), or C33-A (cervical carcinoma)] or a human tissue blot [containing 2 μg (per lane) of poly(A)$^+$ RNA isolated from the indicated tissues (CLONTECH)] was screened with probes corresponding to the full-length E2F6 ORF or a 1,052-bp *Xmn*I-*Sac*I fragment from the 3' untranslated region. These fragments were labeled with [α-^{32}P]dCTP by using the Prime-It II kit (Stratagene). The blots were hybridized for 18 h at 65°C in 0.5 M sodium phosphate, pH 7.5/1 mM EDTA, pH 8.0/5% SDS/1% BSA and washed three times with 1X SSC/0.1% SDS at 65°C before autoradiography.

Polyclonal Antibody Production and Western Blotting. The full-length 6X His-tagged human E2F6 protein (amino acids 9-275) was expressed in bacteria, purified over a Ni^{2+}-nitrilotriacetic acid-agarose resin (Qiagen) and used to immunize mice. Western blotting was performed as described by Moberg *et al* (Moberg et al. 1996).

Transient Transfection and *in Vitro* Assays. Cells were grown under standard conditions in DMEM supplemented with 10% fetal calf serum. Transient transfections were performed as described (Ausubel et al 1988). For the immunoprecipitation and gel retardation assays, transfections were conducted with 10 μg of each of the indicated plasmids plus pCMV-neo-Bam to give a total of 30 μg. Gel shift assays were carried out as described (Moberg et al. 1996) with unlabeled cell extracts normalized for total protein concentration. For immunoprecipitations, the cells were labeled with 250 μCi of

[^{35}S]methionine Express labeling mix (NEN) in methionine-free medium (GIBCO/BRL) for 3.5 h. Immunoprecipitations were performed with the following antibodies: 12CA5 [anti-hemagglutinin (HA) tag], KH20 [anti-E2F1 (Helin et al. 1992)], LLF4-1 [anti-E2F4 (Verona et al. 1997)], sc-610x [anti-DP1 (Santa Cruz Biotechnology)], sc-829x [anti-DP2 (Santa Cruz Biotechnology)]. Precipitates were resolved on a 10% SDS polyacrylamide gel (SDS-PAGE) and detected by fluorography. For transactivation assays, C33-A cells were transfected in duplicate with 4 μg of pE2F$_4$-CAT, 2 μg of pRSV-luciferase (as an internal control for transfection efficiency), 14 μg of carrier DNA (pBKS+), and the indicated amounts of the pCMV-E2F expression vectors. These transfections were performed in the presence or absence of 3 μg of pCMV-HA-DP2. Within each experiment, the total concentration of CMV expression vector was kept constant by the addition of pCMV-neo-Bam. Chloramphenicol acetyltransferase (CAT) and luciferase assays were conducted as described in Lees *et al* (Lees et al. 1993).

Results

Isolation of cDNAs encoding an E2F family member. At the start of this study, five genes had been identified that encode members of the E2F family of proteins. We have shown previously that these proteins account for a significant proportion of the endogenous E2F-DP complexes, but there must be at least one additional E2F (Moberg et al. 1996). The greatest homology between the known E2F family members maps to the C-terminal half of the DNA binding domain. This contains a stretch of 15 amino acids (QKRRIYDITNVLEGI) that is invariant in the previously identified E2Fs. In an attempt to identify additional E2F family members, we searched the EST database for cDNA clones that encode this motif. As expected, we were able to identify multiple ESTs derived from E2F1, 2, 3, 4, or 5. In addition, this search identified one mouse (GenBank accession no. AA041604/AA050073) and one human (GenBank accession no. AA127210) EST that did not correspond to the known E2Fs. These clones were highly related to one another at the nucleotide level, suggesting that they were mouse and human homologues of the same gene. The QKRRIYDITNVLEGI motif was highly, but not completely, conserved in one of the predicted translation products and almost all of the variation between these two clones was in the third base position of this ORF. These data strongly suggested that this was the correct ORF and that these cDNAs were excellent candidates to encode another E2F.

With both the EST database and standard library screening techniques, we identified multiple overlapping cDNAs that encompassed 2,027 bp of the human gene. This sequence starts within the ORF and extends to the poly(A)$^+$ tail. None of the human

cDNAs diverged from this assembled sequence. We also identified two noncontiguous mouse cDNAs. The first is a 638-bp clone that shares significant homology with the 3' untranslated region of the human gene. The second includes 216 bp of 5' untranslated region followed by the initiating ATG (as judged by the presence of a good Kozak consensus and an upstream in-frame termination codon) and 536 bp of ORF. Sequence comparison suggested that the human cDNA lacks only a short region (18 bp) of 5' coding sequence (data not shown). The overlapping regions of the human and mouse sequence are highly conserved at both the nucleotide (83%) and predicted amino acid (92%) level (Fig. 1A). We therefore concluded that we had identified the mouse and human orthologues of another gene, hereafter designated *E2F6*, that share significant homology with the known E2Fs.

The previously identified E2F proteins have been divided into two distinct subgroups (E2F1 through 3 versus E2F4 and 5) on the basis of differences in their amino acid sequence. The central portion of the E2F6 protein shared considerable homology with the domains of E2F1 through 5 that are known to mediate their dimerization (both the leucine zipper domain and region of homology known as the marked box) and DNA binding properties (Fig. 1B). Within these domains, many of the residues that are absolutely conserved between E2F1 through 5 are also maintained in E2F6. However, at a significant proportion of these conserved residues, *E2F6* had alternative codon usage from that found in the other E2F genes (data not shown). Moreover, when we examined the amino acid positions that are known to distinguish E2F-1 through 3 from E2F4 and 5, the corresponding residue in E2F6 rarely fit into either subgroup (see Fig. 1B). The distinction between E2F6 and the other E2F family members was further underscored by

the degree of sequence variation outside of the DNA binding and dimerization domains. The N-terminal domain of E2F6 was of intermediate length, relative to those of the two E2F subgroups, and resembled neither subclass. More importantly, the E2F6 protein terminated just 42 amino acids beyond the marked box motif (the C-terminal portion of the dimerization domain). As a result this protein lacks the sequences that are known to mediate either the transcriptional activation or pRB, p107, or p130 binding properties of the other E2F proteins. Thus, these findings suggest that E2F6 represents a third subclass of the E2F protein family that is likely to display distinct properties from the previously identified members.

E2F6 is Widely Expressed in Vivo. E2F1 through 5 are all expressed in a wide variety of tissues (Helin et al. 1992; Kaelin et al. 1992; Ivey-Hoyle et al. 1993; Lees et al. 1993; Beijersbergen et al. 1994; Ginsberg et al. 1994; Hijmans et al. 1995). We were therefore interested to establish the expression pattern of E2F6. Initially, we isolated poly(A)$^+$ RNA from the indicated human cell lines and screened them for the presence of the E2F6 mRNA by northern blotting using a probe derived from the coding sequence (Fig. 2A). In these and every other cell line examined, this probe hybridized with similar stringency to two distinct messages. To date, each of the E2F6 cDNAs that we have identified corresponds to a single common transcript. It was therefore unclear why the E2F6 probe detected two different mRNAs. To address this issue, we rescreened these northern blots with a probe derived from the 3' untranslated region. This second probe also hybridized to the same two transcripts (data not shown), indicating that they must both contain at least some of the sequences from both the coding and 3' untranslated region of our E2F6 cDNA. We also examined the expression of E2F6 in human tissues.

For these experiments, we screened a human tissue blot (CLONTECH) with the probe corresponding to the E2F6 coding region (Fig 2A). As with the cultured cells, we detected two E2F6 transcripts in every tissue examined. We therefore that E2F6 will be expressed in most if not all cell and tissue types. At this time, we are unable to explain the structural difference(s) between the two mRNAs.

Given the existence of the two E2F6 transcripts, we could not rule out the possibility that alternative splicing could give rise to protein products with two distinct C-termini, one that corresponds to that encoded by our identified cDNA clones and one that more closely resembles the domain structure of the previously identified E2F proteins. To address this issue, we raised polyclonal antiserum against the predicted human E2F6 protein. Control experiments confirmed that the antiserum recognized epitopes throughout the E2F6 protein, but did not cross-react to any of the other known E2Fs (data not shown). When tested on western blots, the antiserum detected a single 35-kDa protein in all tested cell lines that migrated slightly faster than an HA-tagged version of the E2F6 protein produced by transient transfection (Fig. 2B and data not shown). We therefore conclude that the E2F6 protein is expressed *in vivo* and it exists predominantly in the form predicted by the cDNA clones.

E2F6 Displays Low-Affinity E2F DNA Binding Activity. Given the unusual structure of E2F6, we wished to establish whether this protein retained any of the properties of the known E2F family members. All previously identified E2Fs can bind to either of the human DPs and this heterodimerization is known to be a prerequisite for the high-affinity DNA binding. We therefore initiated our analysis by comparing the ability of E2F1, 4, and 6 to bind to known E2F-associated proteins. For these experiments, C33-

A cells were transiently transfected with eukaryotic expression vectors encoding E2F1, E2F4 or an HA-tagged version of E2F6 in the presence of absence of the DP (either DP1 or DP2) or pocket (pRB, p107, or p130) proteins. The transfectants were labeled with [^{35}S]methionine and then subjected to immunoprecipitation with the indicated antibodies (Fig. 3A and data not shown). Although there was considerable variation in the efficiency of the individual transfections, these experiments allowed us to assess the DP and pocket protein binding properties of the individual E2F proteins. A monoclonal antibody specific for the HA tag was able to recover either DP1 or DP2 in approximately stochiometric amounts with HA-E2F6 (Fig. 3A, compare lanes 18 and 21 with lanes 3 and 11 and lanes 6 and 14). This confirmed that E2F6 is able to heterodimerize with the DP proteins despite the sequence variation within its presumed dimerization domain. Consistent with previous studies, the E2F1/DP1 (Fig. 3A, lanes 4 and 5) and E2F4/DP1 (Fig. 3A, lanes 12 and 13) complexes were able to associate with pRB and p107 when overexpressed. In contrast, we did not detect any interaction between the HA-E2F6/DP complexes and any of the three pocket proteins (Fig. 3A, lanes 19 and 20 and data not shown). This finding is consistent with the absence of a pocket protein binding motif within the E2F6 protein sequence.

After establishing that E2F6 can associate with the DP proteins, we next examined the DNA binding activity of these heterodimers. Transient transfection was used to generate the relevant E2F complexes, exactly as described above, and the resultant cell extracts were measured for total protein content. These were then tested in gel retardation assays with the consensus E2F site and equivalent input levels of total protein (Fig. 3B). Consistent with previous studies, E2F1 and E2F4 were unable to bind to DNA in the

absence of a cotransfected DP protein (Fig. 3B, lanes 3 and 8). In a similar manner, we were unable to detect any increase in the levels of E2F DNA binding activity in cells that were transfected with HA-E2F6 alone (Fig. 3B, lane 13). In the same assay, E2F1, 4, and 6 were able to bind to DNA when associated with either DP1 (Fig. 3B, lanes 4, 9, and 14) or DP2 (Fig. 3B, lanes 7, 12, and 17). However, we consistently detected less E2F DNA binding activity in extracts derived from cell transfected with HA-E2F6 rather than E2F1 or E2F4 (Fig. 3B, compare lanes 14-17 with lanes 4-7 and 9-12). Given this finding, we also assessed the expression levels of these E2Fs in western blots (Fig. 3B). Because the blots were probed with different antibodies, we cannot make definitive conclusions about the relative levels of these proteins. However, these antibodies have similar avidities for their respective antigens (our unpublished observations), suggesting that E2F1 and E2F4 are not expressed at significantly higher levels than HA-E2F6. This suggests that HA-E2F6 containing complexes have a lower affinity for the consensus E2F site relative to complexes containing either E2F1 or E2F4. This low DNA binding activity was observed with multiple independent transfections and was not improved by the removal of the HA tag (data not shown). Given these observations, we also tested the E2Fs for their ability to bind to other E2F binding sites (data not shown). The DNA binding activity of E2F6/DP did increase when we used the probe TTTCCCGCC(A/T)(A/T)(A/T). However, this site was previously identified by site selection assays as the preferred recognition sequence of complexes containing E2F1, 2, 3, or 4, and these species also have a higher affinity for this sequence than for any other E2F site (B.F., unpublished data). In fact, relative to the other E2F species, E2F6/DP complexes bound less well to each of the five probes tested. We therefore conclude that

E2F6 has a lower affinity for DNA, at least when associated with either of the known DP proteins.

E2F6 is Unable to Activate the Transcription of E2F-Responsive Genes. E2F6 lacks the C-terminal sequences that are known to mediate the transcriptional activity of E2F1 through 5. However, its shortened C-terminal domain is highly charged and contains a significant proportion of acidic residues (22%), suggesting that it might activate transcription via this alternative motif. To test this hypothesis, we transiently transfected C33-A cells with a chimeric reporter construct, $E2F_4$-CAT, in which the expression of the CAT gene is controlled by a minimal promoter containing four consensus E2F sites upstream of the E1B TATA box (Helin et al. 1993) and increasing amounts of the eukaryotic expression vectors encoding E2F1, E2F4, or HA-E2F6 (Fig.4A). These transfections were conducted in either the absence or presence of CMV-DP2 as indicated. E2F1 and E2F4 both substantially increased the activation of this reporter. In contrast, E2F6 did not bring about any increase in the level of E2F transcriptional activity, either in the absence or presence of cotransfected DP proteins (Fig 4A). Instead, the increasing input levels of CMV-E2F6 steadily inhibited the ability of the endogenous E2F complexes to activate this reporter (Fig. 4B). We did not see any inhibition of reporter activity when the E2F sites were deleted, indicating this repression was specific for E2F-responsive reporters (data not shown). This inhibition was observed in either the absence or presence of an excess of exogenously expressed DP protein (Fig. 4B and data not shown).

After finding that E2F6 can block the activity of the endogenous E2F complexes, we wanted to establish whether it could also inhibit a cotransfected E2F. For this

experiment, C33-A cells were transiently transfected with the $E2F_4$-CAT reporter in either the absence or presence of CMV-E2F1 (50 ng) and increasing amounts of CMV-E2F6 (Fig 4C). At this input level, E2F1 increased the activation of this reporter by approximately 6-fold. This was effectively inhibited by increasing input levels of CMV-E2F6. Exactly as described above, this repression was observed in either the absence or presence of cotransfected DP (data not shown). We therefore conclude that E2F6 is unable to activate transcription and, at least when overexpressed, can inhibit the transcriptional activity of the other E2F species.

Discussion

E2F plays a pivotal role in the regulation of cellular proliferation by controlling the expression of genes that are essential for either entry into, or passage through, the cell cycle. This activity is extremely complex, arising from the combined action of multiple E2F/DP heterodimers. Although there are differences in their cell cycle regulation, the previously identified E2F/DP species can all display the same transcriptional properties. In the presence of an associated pocket protein, they each behave as transcriptional repressors. Once released from the pocket proteins, these same E2F/DP species can activate transcription. Although the DP moiety is required for activity, the transcriptional activation and pocket protein binding properties of the E2F/DP complexes are entirely dependent upon sequences within the E2F protein.

Herein we describe the cloning and characterization of the human and murine orthologues of a gene that contains the most conserved domains of the known E2F family members, including the core sequences required for DNA binding and dimerization. Functional studies confirm that the human protein can dimerize with either DP1 or DP2 and the resultant complexes can bind to DNA in a sequence-specific manner. These findings confirm that this protein is a genuine member of the E2F family, and we have, therefore, named it E2F6. Despite this designation, our data indicate that this protein functions in a different manner from the other E2F family members. (i) E2F6-containing complexes bind to DNA significantly less well than the other E2F/DP species. Although we have yet to calculate dissociation constants for these interactions, our preliminary studies suggest that E2F6-containing complexes recognize the same target sequences as

the other E2F species but they bind to these sites with at least a 5-fold lower affinity. (ii) E2F6 lacks the domains that are known to mediate the transactivation and pocket protein binding properties of the other E2Fs and it is unable to perform either of these functions. (iii) Overexpressed E2F6 is able to block the transcriptional activity of either cotransfected E2F1 or the endogenous E2F complexes. Although the mechanism of this repression is unclear, our data indicate that transcriptional inhibition is not due solely to the sequestration of DP from the other E2F family members by abnormally high levels of E2F6.

Many other transcription factor families include one or more members that behave as transcriptional inhibitors but there is considerable variation in the mechanism by which repression is mediated. The Id proteins of the myogenic basic helix-loop-helix family, the I-POU protein of the POU domain family, and the CHOP protein of the C/EBP-like family lack the sequences necessary for high-affinity DNA binding and, therefore, function in a DNA-independent manner (Benezra et al. 1990; Treacy et al. 1991; Ron and Habener 1992). Instead, they are able to inhibit transcription by binding to the essential heterodimeric partners of the positively acting family members and forming nonfunctional complexes. There are also several examples of inhibitory proteins that repress transcription in a DNA-binding-dependent manner. In this case, inhibition can be mediated by either exclusion of other family members from the DNA or through the recruitment of cellular factors that actively inhibit transcription. One example of this latter case is the Mad-Max complex that has recently been shown to mediate repression in a sequence-specific manner through its ability to recruit histone deacetylase activity to its target genes (Pazin and Kadonaga 1997).

Because the E2F proteins function as part of a heterodimeric complex, it is possible to envisage that the repressive effects of E2F6 could be mediated by any of the mechanisms described above. Although we cannot rule out any of these models, two observations suggest that E2F6 is unlikely to act solely through the sequestration of the DP proteins. (i) Unlike the other transcriptional repressors that act by this mechanism, E2F6 retains the ability to bind to DNA. (ii) At least as observed in our overexpression assays, addition of excess DP protein does not prevent E2F6 from inhibiting the activity of the other E2F complexes. This strongly suggests that the observed repression is dependent upon an additional function(s) of the E2F6 protein. DP binding might still be required for this repression as an essential component of an actively repressive complex.

Clearly, the experiments described above do not allow us to address the mechanism of action of the endogenous E2F6 species. In fact, it is still unclear whether or not E2F6 functions as a repressor *in vivo*. It is possible to envisage alternate models for E2F6 function. For example, the DP proteins are known to be present in excess relative to the E2Fs *in vivo* and E2F6 could exist to chaperone this pool of "unbound" DP. If correct, the "chaperone" model yields two clear predictions about the endogenous E2F6 protein. (i) It should exist at sufficiently high levels in vivo to be able to bind to any DP protein that is not associated with E2F1 through 5. (ii) E2F6 should have a lower affinity/avidity for the DPs than any of the other E2F family members to ensure the efficient transfer of the DP from E2F6 to E2F1 through 5. Clearly, the "repressor" model of E2F6 action also yields testable predictions. Regardless of whether or not its repressive properties are dependent or independent of its ability to bind to DNA, E2F6 should have a similar or greater affinity for the DP proteins than the other E2Fs. Depending on its mechanism of

action, it could also be present at either greater (if it acts through the sequestration of DP) or similar/lower (if it regulates transcription in a sequence specific manner) levels than the other E2Fs *in vivo*. A direct comparison of the relative affinity/avidity of the individual E2F family members for DP should help us to distinguish between these "chaperone" and "repressor" models, as will information about the relative abundance and DNA binding activity of the endogenous E2F6 protein. Although we have yet to establish the true physiological role of E2F6, its broad expression pattern suggests that it makes an important contribution to the regulation of E2F activity *in vivo*.

Acknowledgements

We are grateful to members of the Lees laboratory for helpful discussion throughout the course of this study and to Patrick Humbert and Steve Bell for critical reading of the manuscript. This work was supported by a grant from the National Institutes of Health. J.A.L. is a recipient of the Professor Irwin Sizer and Helen Sizer Career Development Professorship.

1A

```
hE2F6   ......HEKLPSLLLDPTEETVRRRCRDPINVEGLLPSKIRINLEDNVQYVSMRKALKVKRPRFDVSLVYLTRK
mE2F6   MSQQRTARRQPSLLLvPTAQTVRRRCRDPINVEGNLPSKIRINLEENVQYVSMRKALKVKRPRFDVSLVYLTRK

hE2F6   FMDLVRSAPGGILDLNKVATKLGVRKRRVYDITNVLDGIDLVEKKSKNHIRWIGSDLSNFGAVPQQKKLQEELS
mE2F6   FMDLVRSAPACILDLNKVATKLGVRKRRVYDITNVLDGIELVEKKSKNHIRWIGSDLNNFGAAPQQKKLQAELS

hE2F6   DLSAMEDALDELIKDCAQQLFELTDDKENERLAYVTYQDIHSIQAFHEQIVIAVKAPAETRLDVPAPREDSVTV
mE2F6   DLSAMEDALDELIKDCAQQLLELTDDKENE.........

hE2F6   HIRSTNEPIDVYLCEVEQGQTSNKRSEGVGTSSSESTHPEGPEEEENPQQSEELLEVSN*
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1B

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E2F2                                                    MLQGPR.ALASAAGQTPKVVPAMSPTELWPSGLSSPQLCPATATYYTPL
E2F3    MRKGIQPALEQYLVTAGGGEGAAVVAAAAAASMDKRALLASPGFAAAAAAAAPGAYIQILTTNTSTTSCSSSLQSGAVAA..GPL
E2F1                                                    MALAGAPAGGPCAPALEALLGAGALRLLDSSQIVIISAAQDASAP

E2F2    YPQTAPPAAAPG.....TCLDATPHGPEGQV.............VRCLPAGRLPAKRKLDLEGIGRPVVPE.FPTPKGKC.IRVDGLP
E2F3    LPSAPGAEQTAG.....SLLYTTPHGPSSRAGLLQQPPALGRGGSGGGGGPPAKRRLELGESGHQYLSDGLKTPKGKGRAALRSPD
E2F1    PAPTGPAAPAAGPCDPDLLLFATPQAPRPTPS..........APRPALGRPPVKRRLDLET.DHQYLAESSGPARGRGR......H
E2F6          HEKLPS.....LLLDPTEETVRRRC..........RDPINVEGLLPSKIRINLED.................NVQYVS
E2F4                                                                                    MAEAGPQAPP
E2F5                                                                  MAAAEPASSGQQAPAGQGQGQRPPPQPPQAQAPQPPP

                                                  DNA binding domain
E2F2    SPKTPKSPGEKTRYDTSLGLLTKKFIYLLSESEDGVLDLNWAAEVLDV.QKRRIYDITNVLEGIQLIRKKAKNNIQWVGRGM.FED
E2F3    SPKTPKSPSEKTRYDTSLGLLTKKFIQLLSESEDGVLDLNWAAEVLDV.QKRRIYDITNVLEGIQLIRKKAKNNIQWMGCSL.SED
E2F1    PGKGVKSPGEKSRYETSLNLTTKRFLELLSHSADGVVDLNWAAEVLKV.QKRRIYDITNVLEGIQLIAKKSKNHIQWLGSH...TT
E2F6    MRKALK..VKRPRFDVSLVYLTRKFMDLVRSAPGGILDLNKVATKLGVR.KRRVYDITNVLDGIDLVEKKSKNHIRWIGSDL.SM.
E2F4    PP......GTPSRHEKSLGLLTTKFVSLLQEAKDGVLDLKLAADTLAVRQKRRIYDITNVLEGIGLIEKKSKNSIQWKGVGPGCNT
E2F5    PPQLGGAGGGSSRHEKSLGLLTTKFVSLLQEAKDGVLDLKAAADTLAVRQKRRIYDITNVLEGIDLIEKKSKNSIQWKGVGAGCNT

E2F2    PTRPGKQQQLGQELKELMNTEQALDQLIQSCSLSFKHLTEDKANKRLAYVTYQDIRAVGNFKEQTVIAVKAPPQTRLEVPDR....
E2F3    GGMLAQCQGLSKEVTELSQEEKKLDELIQSCTLDLKLLTEDSDNQRLAYVTYQDIRKISGLKDQTVIVVKAPPETRLEVPDS....
E2F1    VGVGGRLEGLTQDLRQLQESEQQLDHLMNICTTQLRLLSEDTDSQRLAYVTCQDLRSIADPAEQMVMVIKAPPETQLQAVDS....
E2F6    FGAVPQQKKLQEELSDLSAMEDALDELIKDCAQQLFELTDDKENERLAYVTYQDIHSIQAFHEQIVIAVKAPAETRLDVPAP....
E2F4    REIADKLIELKAEIEELQQREQELDQHKVWVQQSIRNVTEDVQNSCLAYVTHEDI..CRCFAGDTLLAIRAPSGTSLEVPIPE.GL
E2F5    KEVIDRLRYLKAEIEDLELKERELDQQKLWLQQSIKNVMDDSINNRFSYVTHEDI..CNCFNGDTLLAIQAPSGTQLEVPIPEMGQ

E2F2    .TEDNLQIYLKSTQGPIEVYLCPEE......VQEPDSPSEEPLPSTSTLCPSPD.......SAQPSSSTDPSIMEPTASSVPAPAP
E2F3    .IE.SLQIHLASTQGPIEVYLCPEE......T.ETHSPMKTNNQDHNGNIPKPA.......SKD.........LASTNSG......
E2F1    .SE.NFQISLKSKQGPIDVFLCPEE......TVGGISPGKTPSQEVTSEEENRATDSATIVSPPPSSPPSSLTTDPSQSLLSLEQE
E2F6    .REDSVTVHIRSTNEPIDVYLCEVE......QGQTSNKRSEGVGTSSSESTHPE.......GPEEEENPQQSEELLEVSN*
E2F4    NGQKKYQIHLKSVSGPIEVLLVNKEAWSSPPVAVPVPPPEDFVQSPSAVSTPPPLPKPALAQSQEASRPNSPQLTPTAVPGSAEVQ
E2F5    NGQKKYQINLKSHSGPIHVLLINKESSSSKPVVFPVPPPDDLTQPSSQSLTPVTPQKSSMA.....................

E2F2    TPQQAPPPPSLVPLEATDSLLELPHPLLQQTEDQFLSPTLACSS.....................................
E2F3    ...HSDCSVSMGNL....SPLASPANLLQQTEDQIPSNL...EG.....................................
E2F1    PLLSRMGSLRAPVDEDRLSPLVAADSLLEHVREDFSGLL...PE.....................................
E2F6
E2F4    GMAGPAAEITVSGGPGTDSKDSGELSSLPLGPTTLDTRPLQSSALLDSSSSSSSSSSSSSNSNSSSSSGPNPSTSFEPIKADPTGV
E2F5    ..............TQNLPEQHVSE.............RSQALQQTSATDISSGS.....................................

                                                                  Pocket protein binding
E2F2    ..................................PLISFSPSLDQDDYLWGLEAGEGISDLFDSYDLGDLLIN*
E2F3    ..................................PFVNLLPPLLQEDYLLSLGEEEGISDLFDAYDLEKLPLVEDFMCS*
E2F1    ..................................EFISLSPPHEALDYHFGLEEGEGIRDLFD.CDFGDLTPLDF*
E2F6
E2F4    LELPKELSEIFDPTRECMSSELLEELMSSEVFAPLLRLSPPPGDHDYIYNLDESEGVCDLFDLPVLNL*
E2F5    ................ISGDIIDELMSSDVF.PLLRLSPTPAD DYNFNLDDNEGVCDLFDVQILNY*
```
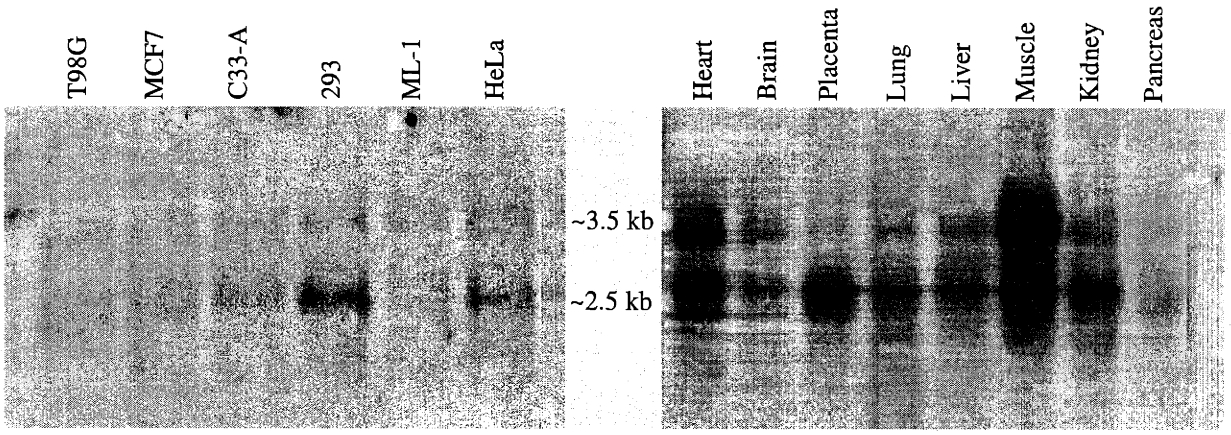

Figure 2

A.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MCF7
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ML-1
HeLa
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Liver
Muscle
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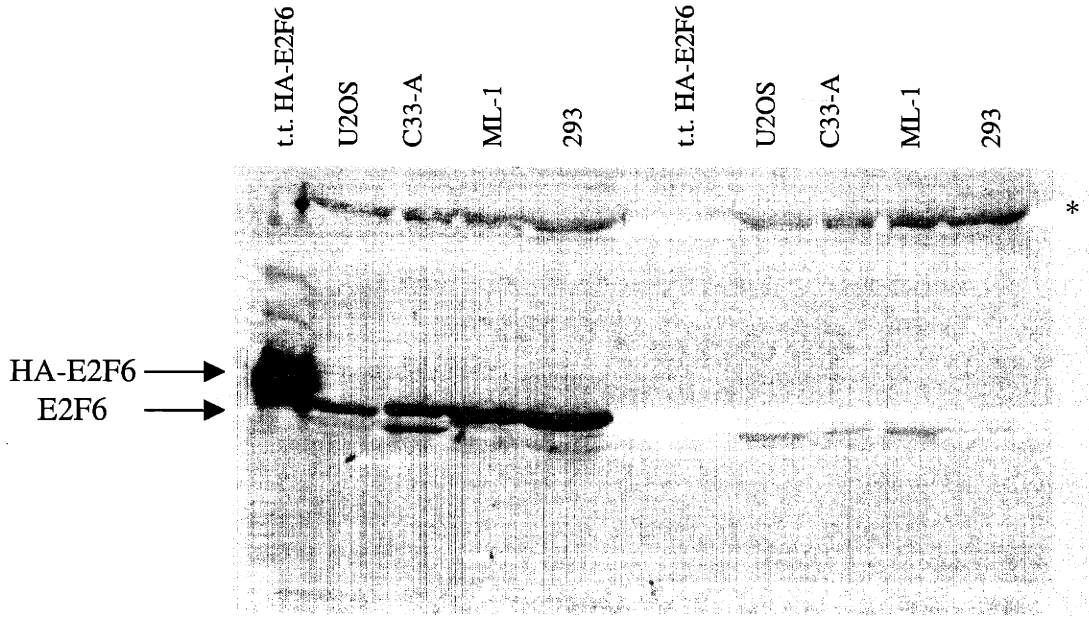
~2.5 kb

B.


t.t. HA-E2F6
U2OS
C33-A
ML-1
293
t.t. HA-E2F6
U2OS
C33-A
ML-1
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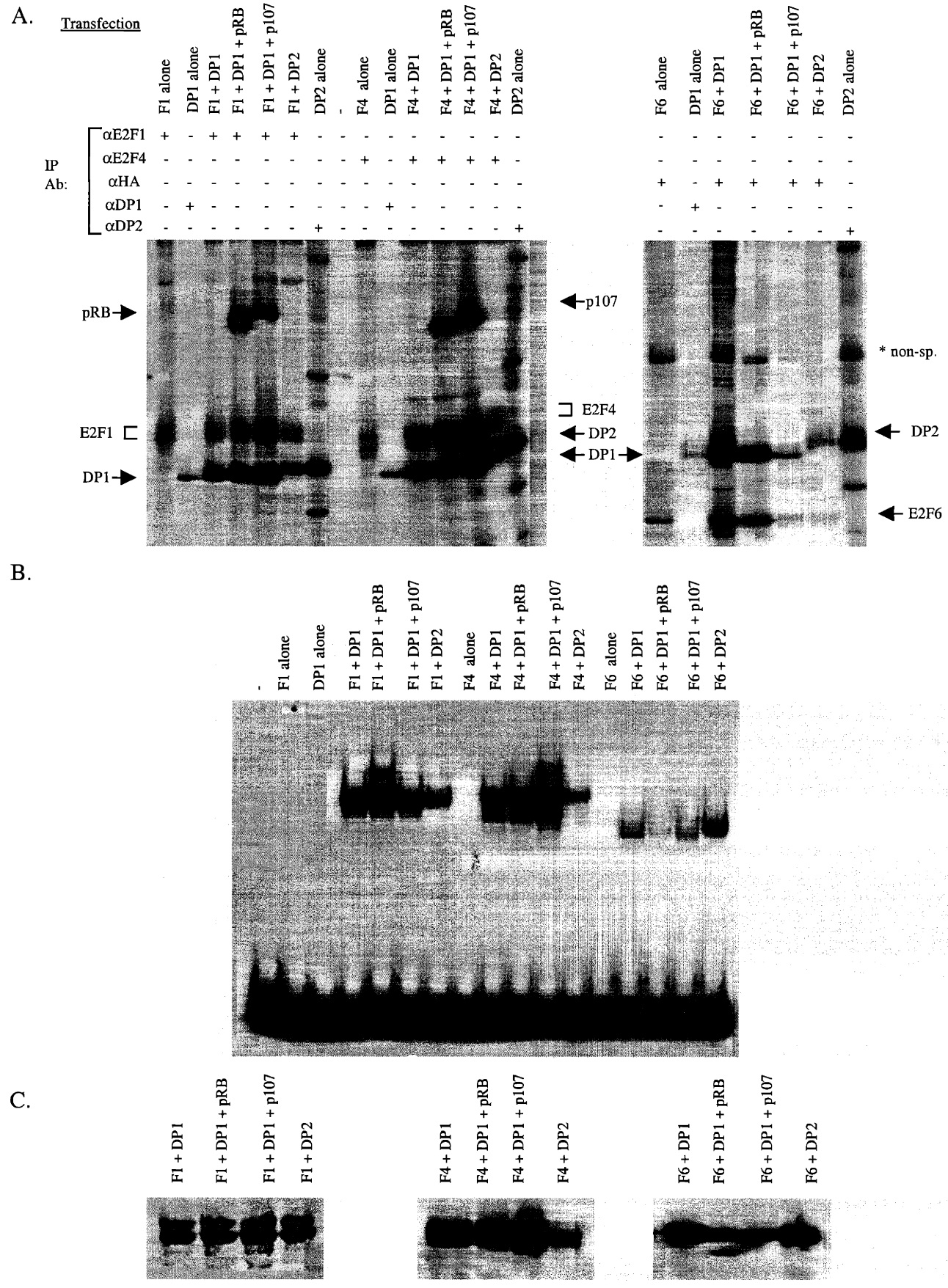
HA-E2F6
E2F6
α-E2F6
Normal mouse

Figure 3


A.
Transfection
F1 alone
DP1 alone
F1 + DP1
F1 + DP1 + pRB
F1 + DP1 + p107
F1 + DP2
DP2 alone
-
F4 alone
DP1 alone
F4 + DP1
F4 + DP1 + pRB
F4 + DP1 + p107
F4 + DP2
DP2 alone
F6 alone
DP1 alone
F6 + DP1
F6 + DP1 + pRB
F6 + DP1 + p107
F6 + DP2
DP2 alone
IP Ab:
αE2F1
αE2F4
αHA
αDP1
αDP2
pRB
E2F1
DP1
p107
E2F4
DP2
DP1
* non-sp.
DP2
E2F6
B.
-
F1 alone
DP1 alone
F1 + DP1
F1 + DP1 + pRB
F1 + DP1 + p107
F1 + DP2
F4 alone
F4 + DP1
F4 + DP1 + pRB
F4 + DP1 + p107
F4 + DP2
F6 alone
F6 + DP1
F6 + DP1 + pRB
F6 + DP1 + p107
F6 + DP2
C.
F1 + DP1
F1 + DP1 + pRB
F1 + DP1 + p107
F1 + DP2
F4 + DP1
F4 + DP1 + pRB
F4 + DP1 + p107
F4 + DP2
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F6 + DP1 + p107
F6 + DP2

Figure 4

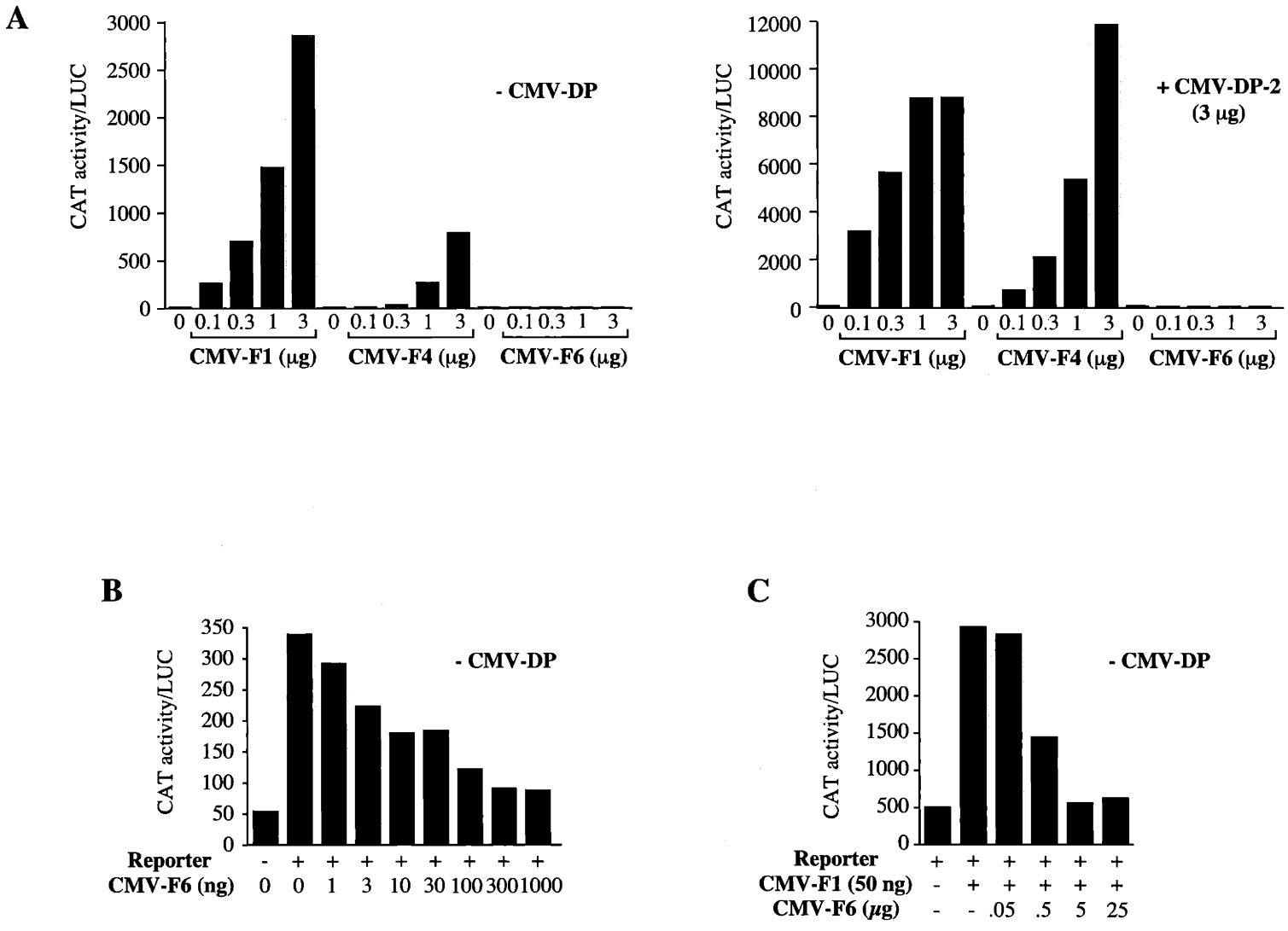
A
CAT activity/LUC
- CMV-DP
+ CMV-DP-2 (3 μg)
0 0.1 0.3 1 3 0 0.1 0.3 1 3 0 0.1 0.3 1 3
CMV-F1 (μg) CMV-F4 (μg) CMV-F6 (μg)
B
- CMV-DP
Reporter - + + + + + + + +
CMV-F6 (ng) 0 0 1 3 10 30 100 300 1000
C
- CMV-DP
Reporter + + + + + +
CMV-F1 (50 ng) - + + + + +
CMV-F6 (μg) - - .05 .5 5 25

Figure Legends

Fig. 1 E2F6 is another member of the E2F family. (A) Amino acid sequence comparison of human versus murine E2F6. (B) Amino acid sequence comparison of the human E2F proteins. Domains responsible for DNA binding, dimerization (leucine zipper, marked box), and pocket protein binding are indicated. Conserved residues are denoted in boldface type.

Fig.2 Expression patterns of E2F6. (A) Northern blot analysis of poly(A)$^+$ RNA isolated from the indicated cell lines and tissues (human tissue blot from CLONTECH), screened with a probe derived from the full-length E2F6 coding sequence. (B) Western blot analysis of extracts derived from C33-A cells transiently transfected with pCMV-HA-E2F6 (200 ng) and from untransfected U2OS, C33-A, ML-1, and 293 cells (50 μg). The positions of HA-E2F6 and endogenous E2F6 proteins are indicated by arrows. The band running beneath HA-E2F6 is a degradation product of this transfected protein (data not shown). The asterisk denotes a nonspecific band.

Fig. 3 Dimerization and DNA binding properties of E2F6. (A) C33-A cells were transiently transfected with expression vectors encoding E2F1, E2F4, or HA-E2F6 in the presence or absence of DP (DP1 or DP2) or pocket proteins (pRB, p107) and immunoprecipitated with the indicated antibodies. (B) C33-A cells were transiently transfected with the identical combinations of expression vectors as in A. Gel shift assays were carried out on the indicated unlabeled cell extracts (1.5 μg of total protein per lane) by using the consensus E2F site from the adenoviral E2 promoter

(TTTCGCGCCCTTT). Western blot assays were carried out on the same unlabeled extracts (300 ng of total protein per lane) by using monoclonal antibodies against E2F1 (KH95), E2F4 (LLF4-1), or the HA tag (12CA5). Gel retardation assays have also been conducted by using the additional E2F sites TTTCCCGCCTTT, TTTCCCGCCAAA, TTTCCCGCGTGT, or ATTCCCGCGCTTT with similar differences in affinity.

Fig. 4 Effects of E2F6 on transactivation of an $E2F_4$-CAT reporter. C-33A cells were transiently transfected in duplicate with 4 μg of $E2F_4$-CAT, 2 μg of Rous sarcoma virus-luciferase (as an internal control), 14 μg of carrier DNA (pBKS+ and pCMV-neo-Bam) in the absence or presence of 3 μg of pCMV-HA-DP2 plus pCMV-E2F expression vectors as indicated. CAT and luciferase activity were determined 24 hours after transfection. The values shown are the average of duplicate transfectants for representative experiments.

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Chapter 3

E2F6 is a component of the mammalian Bmi1-containing polycomb complex

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Author's contribution: Figures 1, 2, 3, and 4

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Abstract

The E2F transcription factors play a key role in the regulation of cellular proliferation and terminal differentiation. E2F6 is the most recently identified and the least well understood member of the E2F family. It is only distantly related to the other E2Fs and lacks the sequences responsible for both transactivation and binding to the retinoblastoma protein. Consistent with this finding, E2F6 can behave as a dominant negative inhibitor of the other E2F family members. In this study, we continue to investigate the possible role(s) of E2F6 *in vivo*. We report the isolation of RYBP, a recently identified member of the mammalian polycomb complex (PcG), as a new E2F6-interacting protein. Mapping studies indicate that RYBP binds within the known "repression domain" of E2F6. Moreover, we demonstrate that endogenous E2F6 and PcG proteins, including RYBP, Ring1, MEL-18, mph1, and the oncoprotein Bmi1, associate with one another. These findings suggest that the biological properties of E2F6 are mediated through its ability to recruit the polycomb transcriptional repressor complex.

INTRODUCTION

The E2F transcription factors are a family of genes that play critical roles in the regulation of cellular proliferation and differentiation for review, see (Dyson 1998; Helin 1998). They act by controlling the transcriptional state of genes whose expression is essential for cell cycle progression and DNA synthesis. Deregulated E2F activity has been shown to lead to inappropriate cell cycle entry, transformation and apoptosis. Thus, by understanding the mechanisms by which E2F activity controls the expression of its downstream target genes, we can gain important insights into the processes of cellular proliferation, differentiation and apoptosis.

A total of eight genes have been cloned thus far that encode components of E2F activity reviewed in (Dyson 1998). Their protein products can be subdivided into two families, the E2Fs (1-6) and the DPs (1-2), that heterodimerize to generate functional E2F complexes (Helin et al. 1993; Krek et al. 1993; Wu et al. 1995; Chapter 2). While the presence of a DP protein is required for activity, the functional specificity of the resulting E2F/DP heterodimer is conferred by the E2F moiety (Dyson 1998). The E2F family has been divided into three distinct groups on the basis of both sequence homology and functional properties.

E2F1, E2F2, and E2F3 represent the first E2F subclass. These proteins, when bound to DP, have high transcriptional activity and are sufficient to drive quiescent cells into S phase (Kowalik et al. 1995; Lukas et al. 1996; DeGregori et al. 1997; Verona et al. 1997). In normal cells, the activity of these complexes is controlled by their association with the retinoblastoma protein (pRB), the first tumor suppressor identified (Hiebert et al. 1992). Cell cycle dependent phosphorylation of pRB causes it to dissociate from E2F/DP complexes and this

release correlates with the timing of E2F responsive gene activation *in vivo*. Mouse models for *E2f1* and *E2f3* confirm that these E2Fs play a key role in the activation of E2F target genes and the induction of proliferation arising from either mitogenic signaling or from the loss of the Rb tumor suppressor (Dyson 1998; Ziebold et al. 2001; Appendix C).

E2F4 and E2F5 represent the second subclass of the E2F family. Unlike the first subclass, E2F4 and E2F5 are poor transcriptional activators, and they are unable to induce quiescient cells to enter the cell cycle (Lukas et al. 1996; Muller et al. 1997; Verona et al. 1997). Instead, these E2F proteins are believed to be important for the repression of E2F-responsive genes through their ability to bind to pRB, and the related proteins p107 and p130, and recruit histone deacetylases to the promoters of E2F-responsive genes (Brehm et al. 1998; Dyson 1998). Consistent with this model, the analysis of *E2f4* and *E2f5* mutant mouse strains suggests that these proteins are not required for the regulation of cellular proliferation but play a key role in the terminal differentiation of specific cell types (Lindeman et al. 1998; Gaubatz et al. 2000; Humbert et al. 2000).

Additionally, we (Chapter 2) and others (Morkel et al. 1997; Cartwright et al. 1998; Gaubatz et al. 1998) identified a sixth member of the E2F family. The central portion of the E2F6 protein shares considerable homology with the domains of E2F1 through E2F5 that are known to mediate their heterodimerization and DNA binding properties. However, E2F6 also diverges considerably from the previous E2F subgroups within these domains. The distinction between E2F6 and the other E2Fs is underscored by the degree of sequence variation outside of these domains. The N-terminal domain of E2F6 bears no homology to those of other E2F family members and, most importantly, E2F6 terminates 42 amino acids after the dimerization domain. As a result, E2F6 lacks the sequences that mediate either the

transcriptional activation or pRB, p107 and p130 binding properties of the other E2F proteins. Thus, E2F6 represents a third subclass of the E2F family that is likely to display distinct biological properties.

Using over-expression assays, we have shown that E2F6 can repress the transcription of known E2F-responsive genes (Chapter 2). In these experiments, E2F6 appears to function as a dominant negative inhibitor through competition with other E2F family members. Consistent with these observations, other groups have reported that E2F6 can behave as an active repressor when fused to a heterologous DNA binding domain (Gaubatz et al. 1998). However, the mechanism by which E2F6 represses transcription is not well understood.

We have used a yeast two-hybrid assay to identify E2F6-associated proteins. This analysis yielded RYBP (Ring1 and YY1 binding protein), a known component of the mammalian polycomb complex that binds specifically to the "repression domain" of E2F6. Through the generation of specific immunological reagents, we show that endogenous E2F6 associates with the Bmi1-containing polycomb complex. These data have important implications for our understanding of both E2F and polycomb complex function *in vivo*.

MATERIALS AND METHODS

Yeast-two-hybrid assay. A yeast two-hybrid screen was performed using the system of Vidal and coworkers (Vidal et al. 1996). Briefly, amino acids 9-239 of E2F6 were cloned into the pPC97 vector and the resulting plasmid was transformed into the yeast strain MaV103 by using the lithium acetate method. This strain was transformed with a library derived from activated human T cells and the transformations were plated on synthetic complete (SC) –Leu -Trp plates. Two days later, the transformations were replica-plated onto SC –Leu –Trp –His plates supplemented with 10 mM 3-aminotriazole (3-AT; Sigma) and subsequently replica-plated again onto SC –Leu –Trp –His plates supplemented with 30 mM 3-AT. The prey plasmid was rescued and transformed into bacteria. Plasmids were re-transformed into MaV103 containing pPC97-E2F6 to verify that the 3-AT resistance was conferred by the library plasmid. The inserts in the recovered prey plasmids were sequenced using standard techniques.

Plasmid Construction Full length or truncation mutants of human E2F6, RYBP and mouse Ring1A were generated by PCR and subcloned into pHACMV-neo-Bam or pCMV-neo-Bam. All constructs were verified by sequencing. Full length RYBP was additionally subcloned into pQE30 (Qiagen, Chatsworth, CA). The plasmids pCMV-E2F1, pCMV-E2F2, pCMV-E2F3, pCMV-E2F4, pQE30-E2F6 and pHA-Bmi1 have been described previously (Helin et al. 1993; Moberg et al. 1996; Alkema et al. 1997; Chapter 2).

Antibody production Full-length 6X His-tagged E2F6 (amino acids 1-275) proteins were expressed in bacteria, purified over a Ni^{2+}-nitrilotriacetic acid-agarose resin (Qiagen) and used to immunize mice. The resulting polyclonal antiserum was monitored for its ability to

recognize transfected E2F6 and not E2Fs 1-5 or PcG proteins, by both western blotting and immunoprecipitation. Mice generating E2F6 specific antibodies were sacrificed and the spleens removed. The recovered splenocytes were fused to the SP2/O cell line by a polyethylene glycol-mediated method, generating hybridoma cell lines. After 10 days, the tissue culture supernatants were screened for the ability to detect recombinant E2F6 by an enzyme-linked immunosorbent assay (ELISA). The positive cell lines were single-cell cloned and tested again for E2F6 reactivity /specificity by western blot and immunoprecipitation.

Monoclonal antibodies to 6X HIS-tagged RYBP (1-228) were generated as above. Specificity was determined against transfected RYBP and YAF-2 by western blotting and immunoprecipitation. Anti-Ring1 (ASA8), anti-MEL-18, anti-mph1-SM and anti-Bmi1 (Bmi1-F6) antibodies have been described previously (Alkema et al. 1997; Saurin et al. 1998).

Transient Transfections and Immunoprecipitations Cells were maintained using standard procedures in DMEM containing 10% fetal calf serum. Transient transfections were conducted as described in Chapter 2. For immunoprecipitations, C33-A cells were transfected with 10 μg of each of the indicated plasmids and labeled with 250 μCi of [^{35}S]methionine Express labeling mix (NEN) in methionine-free media (Gibco) for 5 hours. Immunoprecipitations were performed exactly as before (Chapter 2) with either 12CA5 (anti-HA tag), anti E2F6 antibodies (LLF6-1) or anti RYBP antibodies (LLRYBP-1).

For endogenous immunoprecipitations, 10^8 ML-1 cells were lysed in E1A lysis buffer and pre-cleared with protein A-sepharose beads (Pharmacia) at 4 °C for 30 minutes. The lysates were incubated with the indicated antibodies for 1 hour at 4 °C, the immunocomplexes

recovered on protein A-sepharose beads and separated on SDS-PAGE. Western blotting was done as previously described (Chapter 2) using the indicated antibodies.

RESULTS

Isolation of E2F6 interacting proteins To better understand the role of E2F6, we used a yeast two-hybrid approach to identify interacting proteins. A fusion between the GAL4 DNA binding domain (Gal4DBD) and amino acids 9-239 of the E2F6 protein was used as bait to screen cDNAs from an activated human T cell library fused with the Gal4 transcriptional activation domain (Gal4AD). Four independent clones were isolated from the approximately $4x10^5$ transformants on the basis of their ability to interact with E2F6 and not with pRB or DP1 fusion proteins. Sequence analysis showed that two of the four isolated clones encoded overlapping fragments of DP2, a known heterodimeric partner of the other E2F family members. Since we have previously shown that E2F6 and DP2 can co-immunoprecipitate (Chapter 2), this provided strong validation of the screen. The remaining clones were found to contain either the full-length (amino acid 1- 228) or the near full-length (amino acid 12-228) coding sequence of a known, zinc finger-containing protein, RYBP. The murine homologue of RYBP was originally isolated in a yeast two-hybrid screen by virtue of its ability to bind to Ring1A (Garcia et al. 1999). The human and mouse RYBP proteins are completely identical apart from three amino acid substitutions (Q to P, E to D and T to S) at residues 87, 97 and 143 respectively. RYBP is also highly related to YAF-2, a protein that was identified in a yeast two-hybrid screen with YY1 (Kalenik et al. 1997), suggesting that these represent a family of proteins.

A major advantage of our chosen yeast two-hybrid system is the ability to assess the relative strength of protein-protein interactions by measuring the growth of clones in the

presence of 3-aminotriazole (Vidal et al. 1996). Using this assay, we demonstrated that E2F6 had a higher affinity for RYBP than it did for its known interactor, DP2. This strongly suggested that RYBP would be a genuine E2F6 interacting protein.

Interaction between RYBP and E2F6 To confirm that E2F6 and RYBP can interact in mammalian cells, we expressed the full-length RYBP protein in C33-A cells by transient transfection in the presence and absence of a full length E2F6 and then labeled the cells with [^{35}S]methionine. The resultant complexes were recovered and analyzed by SDS-PAGE using monoclonal antibodies that we had generated to specifically recognize E2F6 (LLF6-1) or RYBP (LLRYBP-1). We were consistently able to recover RYBP in the anti-E2F6 immunoprecipitate, albeit at low levels (Figure 1A, lane 4). Moreover, the anti-RYBP antibody was able to co-immunoprecipitate E2F6 (Figure 1A, lane 5). Thus, E2F6 and RYBP can form a complex that is poorly recognized by the anti-E2F6 antibody but is efficiently recovered by the anti-RYBP monoclonal antibody.

Given these observations, we looked for an association between the endogenous E2F6 and RYBP. To address this issue, lysates of ML-1 cells were immunoprecipitated using control, anti-E2F6 (LLF6-1) or anti-RYBP (LLRYBP-1) antibodies, resolved by SDS-PAGE and then immunoblotted with additional anti-E2F6 (LLF6-2) or anti-RYBP (LLRYBP-2) monoclonal antibodies. Consistent with the reduced amount of RYBP recovered in E2F6 immunoprecipitations of transfected cells, we were unable to detect RYBP in E2F6 immunoprecipitations of ML-1 lysates. However, we did recover a significant proportion of E2F6 in the RYBP immunoprecipitate (Figure 1B). We therefore conclude that endogenous E2F6 and RYBP can associate with one another.

Mapping RYBP and E2F6 interaction sites Since the manner in which these two proteins associate will influence their functions *in vivo*, we used a deletion mutant strategy to map the sites of interaction (Figure 2). For these experiments, we generated a panel of RYBP and E2F6 deletion mutants and tested their ability to interact with their full-length partner using transient transfection and co-immunoprecipitation assays. Because anti-E2F6 antibodies appear to destabilize the E2F6•RYBP complex, association was determined by immunoprecipitation through RYBP (of either full-length or HA-tagged deletion mutants) and western blotting for E2F6 (either full-length or deletion mutants). In each case, expression of the RYBP and E2F6 deletion mutants was confirmed by western blotting of the whole cell lysate.

First, we mapped the E2F6 binding site on RYBP (Figure 2A). Since zinc-finger motifs can mediate protein-protein interactions, we began our analysis by deleting the N-terminal 72 amino acids of RYBP. This truncated RYBP protein (73-228) bound to E2F6 with a similar affinity as the full length RYBP (Figure 2A). Since there are no recognizable motifs within the remaining portion (73-228), we generated mutants corresponding to the N-terminal (73-143) and C-terminal (144-228) portions of this fragment. While HA-RYBP (73-143) was unable to associate with E2F6, HA-RYBP (144-228) bound as well as the full-length protein. Unfortunately, additional 5' or 3' deletions of this coding sequence did not yield detectable protein products (data not shown). We therefore generated a panel of C-terminal deletions within the context of the full length RYBP. Although we were now able to generate stable proteins, the absence of the C-terminal 85, 63 or 21 amino acids greatly impaired the interaction between RYBP and E2F6. Thus, the E2F6 binding site is contained

within amino acids 144-228 of RYBP and residues at the very C-terminus of this domain appear to be critical for the interaction of these two proteins.

We next mapped sequences in E2F6 that were required for RYBP binding (Figure 2B). Since the N- and C-terminal domains of E2F6 are completely distinct from those of the other E2F family members, we began our analysis by deleting each of these regions. Given that the C-terminal sequence was absent from the yeast two-hybrid bait, we initially focused our attention on the N-terminal 62 amino acids. Surprisingly, deletion of these residues had no detectable effect on the interaction between E2F6 and RYBP. Additional N-terminal deletion showed that the DNA binding domain (residues 62-128) was also fully dispensable for interaction. Given this finding we examined the consequences of C-terminal deletions. Consistent with the yeast two-hybrid data, the unique C-terminal sequences (residues 241-281) were not required for RYBP binding. However, additional deletion of the "Marked-box" domain (residues 179-281) abolished the interaction between E2F6 and RYBP. Taken together, this analysis indicates that the RYBP binding domain is contained within residues 129-240 and is dependent upon residues between 179 and 240. Consistent with this conclusion, we were able to show that E2F6 (129-240) is sufficient to bind RYBP in this co-immunoprecipitation assay. Moreover, although we were unable to express E2F6 (179-240) in mammalian cells, this domain was sufficient to interact with RYBP in the yeast-two hybrid assay (data not shown). Thus, the RYBP binding domain of E2F6 maps to the dimerization domain, and specifically to the "Marked-box" domain.

The "Marked-box" domain is highly conserved among all members of the E2F family. This raised the possibility that RYBP might interact with one or more of the other E2F proteins. To test this hypothesis, C33-A cells were transfected with expression plasmids

encoding E2F1, E2F2, E2F3, E2F4 or E2F6 in the presence or absence of HA-RYBP. The transfected cells were labeled with [^{35}S]-methionine and then immunoprecipitated with either anti-E2F antibodies (to confirm expression of the E2F proteins) or an anti-HA antibody (to detect RYBP and associated proteins). Although E2F6 was readily detected in the RYBP-immunoprecipitation, we did not recover E2Fs 1-4 (Figure 3). Similarly, RYBP was not detected in anti-E2F1-4 immunoprecipitates (data not shown). We therefore conclude that RYBP interacts specifically with E2F6, and not other members of the E2F family, through its "Marked-box" domain.

E2F6 and the polycomb complex RYBP was originally identified by virtue of its association with Ring1A, a component of the mammalian polycomb complex that has been shown to participate in transcriptional repression (Garcia et al. 1999). Given that E2F6 associates with RYBP *in vivo*, we speculated that it might also associate with other polycomb group (PcG) proteins (Figure 4). First, we sought to establish if E2F6 and Ring1A were associated. C-33A cells were transfected with expression vectors encoding E2F6 and a HA-tagged version of Ring1A (HA-Ring1A). The cells were then labeled with [^{35}S]-methionine and immunoprecipitated with antibodies specific for either E2F6 or the HA tag. Consistent with our hypothesis, Ring1A was recovered in E2F6 immunoprecipitates and E2F6 was co-immunoprecipitated with Ring1A (Figure 4A). Given this finding, we also tested for an interaction between the endogenous E2F6 and Ring1 proteins. Whole cell lysates from human ML-1 cells were incubated with an anti-Ring1A polyclonal antibody (ASA8), the resulting immunocomplexes resolved by SDS-PAGE and then immunoblotted using an anti-E2F6 monoclonal antibody (LLF6-2). No E2F6 protein was recovered by the pre-immune serum but we detected a significant level of E2F6 in the Ring1 immunoprecipitates (Figure

4B). Importantly, we were also able recover Ring1 protein in anti-E2F6 immunoprecipitates (Figure 4B). This strongly suggests that there is a physical association between endogenous E2F6 and Ring1 that can be recovered through the immunoprecipitation of either of these proteins.

One of the best characterized members of the mammalian PcG proteins is Bmi1, an oncogene whose tumorigenic properties are at least partially dependent upon its ability to repress the expression of the $p16^{INK4a}$ and $p19^{ARF}$ tumor suppressors (Jacobs et al. 1999a; Jacobs et al. 1999b). We therefore investigated whether E2F6 also associates with Bmi1. As described above, E2F6 and/or HA-Bmi1 were expressed in cells by transient transfection and association was assessed by immunoprecipitation. These experiments showed that E2F6 did associate with co-transfected Bmi1 (Figure 4C). Finally, we also examined the ability of the endogenous E2F6 to associate with Bmi1 and two additional PcG proteins, MEL-18 and mph1. In each case, western blotting confirmed that E2F6 was present in Bmi1, MEL-18 and mph1 immunoprecipitates, but not in those derived from a variety of control antibodies (Figure 4D and data not shown). Taken together, our data indicate that endogenous E2F6 and Bmi1 proteins associate with one another.

DISCUSSION

E2F6 is the most recently identified member of the E2F family and its sequence is only distantly related to those of the other E2Fs (Morkel et al. 1997; Cartwright et al. 1998; Gaubatz et al. 1998; Chapter 2). In particular, E2F6 lacks the sequences required for either pRB binding or transactivation and it can act as a dominant negative inhibitor of the other E2F family members (Chapter 2). By fusing E2F6 sequences to a heterologous DNA binding domain, Gaubatz et al. mapped its repression function to a C-terminal portion of E2F6 that encompasses the "Marked-box" domain (Gaubatz et al. 1998). Using a yeast two hybrid screen we have now discovered that E2F6 binds to RYBP, a recently isolated member of the mammalian PcG complex. Consistent with this observation, we demonstrate a physical association between endogenous E2F6 and numerous PcG proteins including RYBP, Ring1, MEL-18, mph1 and Bmi1. Since our yeast screen was far from saturating, it will be interesting to establish whether we can find other PcG proteins that bind to E2F6 in this assay. Importantly, RYBP-binding is a specific property of E2F6, and not other members of the E2F family, and it maps to the "Marked-box" domain. Taken together, these data are consistent with E2F6 acting as a component of the mammalian Bmi1-containing polycomb complex *in vivo* and they suggest that E2F6's ability to repress the transcription of E2F-responsive genes is dependent upon its ability to recruit this known transcriptional repressor.

Our observations also raise the possibility that E2F6 will play a key role, beyond E2F regulation, in mediating the changes in transcriptional regulation that are essential for normal developmental patterning. The polycomb group (PcG) was originally identified in

Drosophila as one of two groups of genes whose mutation causes homeotic transformations and alterations in homeotic gene expression patterns (Kennison 1995). Further analysis showed that the PcG group proteins normally act to maintain homeobox (*hox*) genes in a silenced state, while the trithorax complex (trxG) is critical for allowing the transcriptional activation of these genes (Kennison 1995). The PcG proteins are believed to function through the formation of large multisubunit complexes that arise via mutual interactions among conserved protein motifs (Kyba and Brock 1998; Shao et al. 1999). Promoter mapping studies have identified DNA fragments, called polycomb response elements (PREs), that appear sufficient to mediate the silencing effect of the PcG complex but these are several hundred base pairs in length (Strutt et al. 1997; Strutt and Paro 1997). Consequently, the exact mechanism by which PcG proteins interact with the PRE is not well understood but it is believed to involve pleiohomeotic (PHO), a partial homologue of the mammalian Zn finger protein YY1 (Brown et al. 1998).

Many mammalian PcG proteins have also been identified, primarily through their homology with the *Drosophila* PcG proteins or as a result of yeast two-hybrid screens (Gould 1997; Schumacher and Magnuson 1997; Satijn and Otte 1999; Bardos et al. 2000). Mutant mouse models confirm that a number of these mammalian PcG proteins, including Bmi1, M33, MEL-18 and Ring1A, play an important role in controlling the developmental regulation of *hox* gene expression and therefore the formation of the axial skeleton (van der Lugt et al. 1994; Akasaka et al. 1996; Core et al. 1997; del Mar Lorente et al. 2000). Like their *Drosophila* counterparts, these proteins appear to act as multimeric complexes (Alkema et al. 1997). In many cases, however, there are multiple mammalian homologues of each *Drosophila* protein and the phenotypes of the mutant mouse strains suggests that these have

overlapping functions *in vivo* (Bel et al. 1998). Several distinct PcG components have been implicated in DNA binding, including MEL-18 and YY1, but these factors cannot account for the known DNA binding properties of PcG complexes (Kanno et al. 1995; Brown et al. 1998). It has therefore been proposed that additional DNA binding factors must exist in the polycomb complex (Brown et al. 1998).

In addition to its developmental role, Bmi1 has been shown to play a key role in the regulation of senescence and tumorigenicity. Indeed, Bmi1 was originally identified as a common insertion site in Moloney Murine leukemia virus (MoMLV) induced B-cell lymphomas in Eμ*Myc* transgenic mice and only subsequently shown to be a mammalian PcG protein (van der Lugt et al. 1994). The analysis of *Bmi1* mutant mice has yielded considerable insight into the role of Bmi1 in both normal development and tumorigenicity. The loss of Bmi1 results in the derepression of the $p16^{INK4A}$ and $p19^{ARF}$ tumor suppressor genes that are expressed from the *INK4* locus and thereby inhibits cellular proliferation and induces premature senescence (Jacobs et al. 1999a). Consistent with this observation, mouse crosses indicate that the developmental and tumorigenic properties of Bmi1 are both at least partially dependent upon its ability to regulate the expression of the *INK4* locus (Jacobs et al. 1999a; Jacobs et al. 1999b). Although it is widely inferred that Bmi1 mediates the direct transcriptional repression of the *INK4* locus via its participation in the polycomb complex, this has not yet been demonstrated. Moreover, it is currently unclear how the Bmi1 containing polycomb complex is recruited to this locus.

This current study shows that E2F6 is a component of the mammalian polycomb complex and therefore suggests that it will participate in the developmental regulation of gene transcription in a distinct manner from the other E2F family members. Previous studies have

established that $p19^{ARF}$ is an E2F-responsive gene (Bates et al. 1998). This raises the possibility that E2F6 could contribute to the DNA binding specificity of the polycomb complex during the regulation of normal development and tumorigenicity. Generation and analysis of *E2f6* mutant mouse strains will be required to test this hypothesis.

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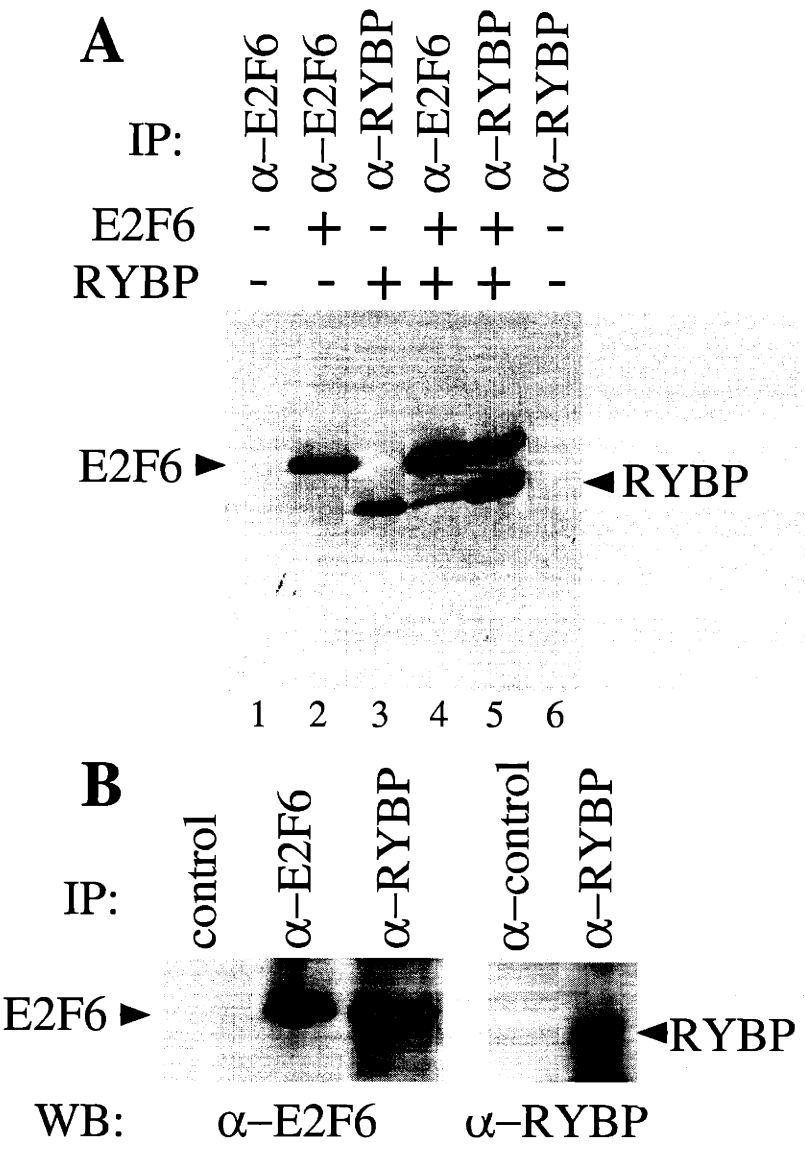
Figure 1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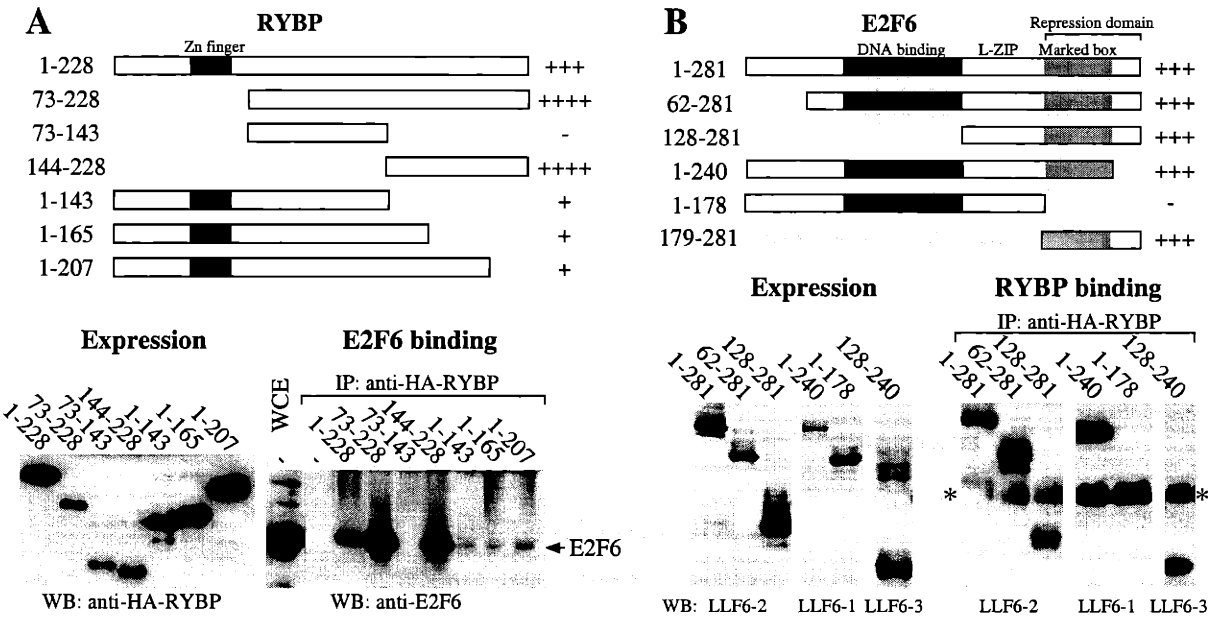
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WCE
IP: anti-HA-RYBP
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73-228
73-143
144-228
1-143
1-165
1-207
E2F6
WB: anti-HA-RYBP
WB: anti-E2F6
B
E2F6
DNA binding
L-ZIP
Repression domain
Marked box
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179-281 +++
Expression
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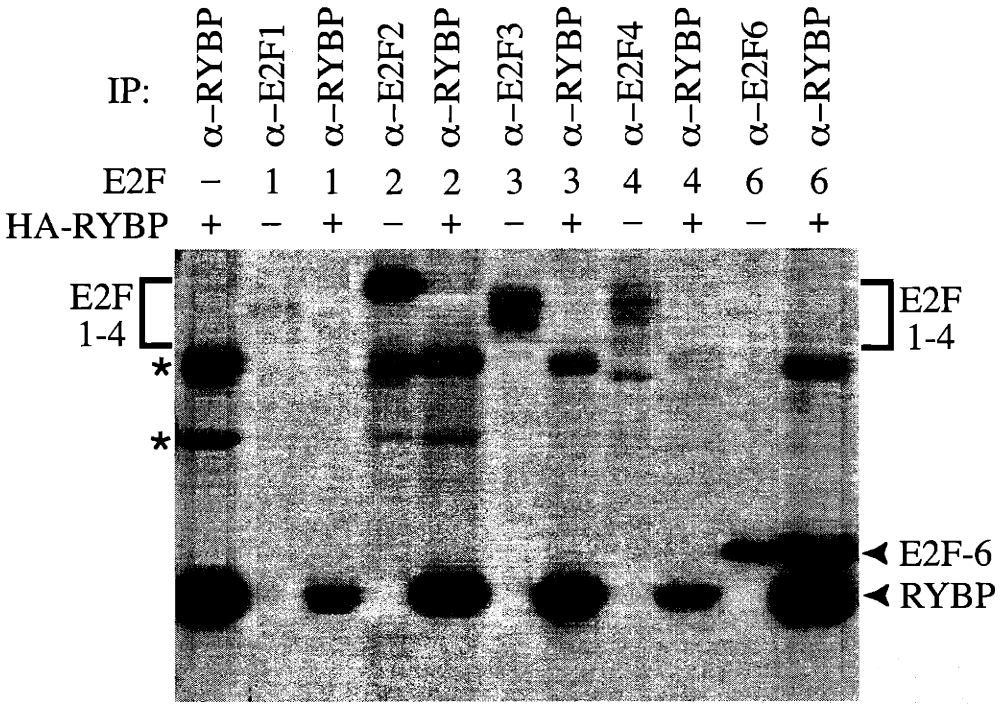
Figure 3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E2F 1-4
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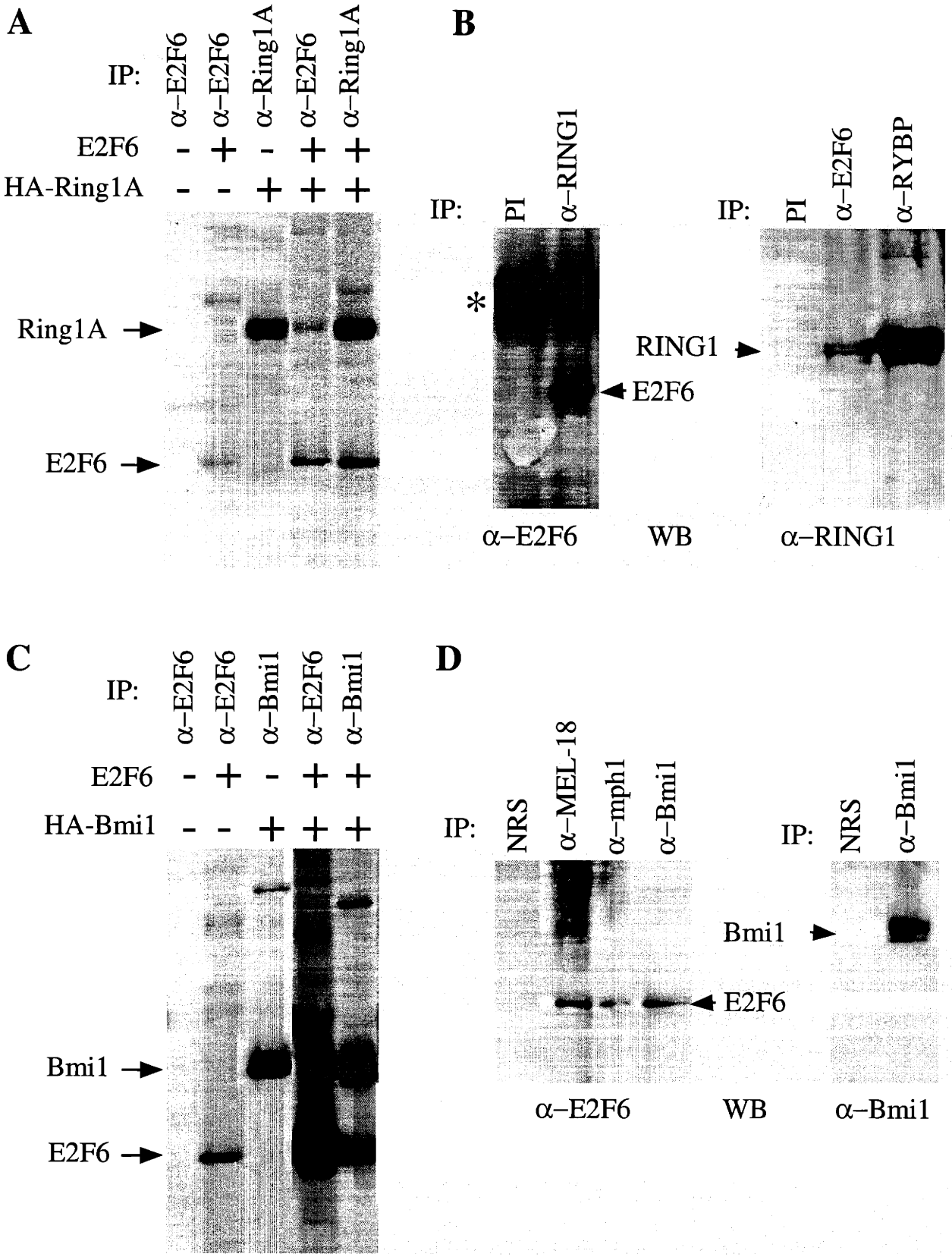
RYBP

Figure 4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α-Bmi1
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NRS
α-Bmi1
Bmi1
E2F6
α-E2F6
WB
α-Bmi1

FIGURE LEGENDS

Figure 1 RYBP is a new E2F6 interacting protein (A) C-33A cells were transiently transfected with expression vectors encoding E2F6 and/or RYBP, labeled with [^{35}S]methionine and immunoprecipitated with the specific monoclonal antibodies LLF6-1 and LLRYBP-1. (B) ML-1 cell lysates were immunoprecipitated with control (12CA5), anti-E2F6 (LLF6-1) or anti-RYBP (LLRYBP-1) antibodies, resolved by SDS-PAGE and immunoblotted with additional anti E2F-6 (LLF6-2) or anti-RYBP (LLRYBP-2) antibodies.

Figure 2 Mapping the E2F6•RYBP interaction domains (A) C33-A cells were transiently transfected with expression vectors encoding full length E2F6 and the HA-tagged RYBP mutants depicted in the diagram. Expression of the HA-RYBP mutants was confirmed by western blotting a fraction of the cell lysate. The remainder was immunoprecipitated with an anti-HA antibody (12CA5) and then immunoblotted with an anti-E2F6 antibody (LLF6-2) to assess the ability of these mutants to bind to E2F6. A summary of the interaction data is shown at the top. (B) The diagram depicts the panel of E2F6 mutants including the location of DNA binding, leucine zipper (L-ZIP), marked box and repression domains. The mutants were transiently transfected into C33-A cells with full length HA-RYBP. The expression of these proteins was confirmed by western blotting a fraction of the cell lysate with the particular anti-E2F6 monoclonal antibody (LLF6-1, LLF6-2 or LLF6-3) that best recognizes each mutant. The remainder of the lysate was immunoprecipitated with an anti-RYBP antibody (LLRYBP-1) and then immunoblotted with the same anti-E2F6 antibody that was

used to confirm its expression. The immunoglobulin light chain is denoted by an asterisk. An interaction summary is depicted at the top.

Figure 3 RYBP interacts specifically with E2F6. C33-A cells were transiently transfected with expression vectors for E2F1, E2F2, E2F3, E2F4 or E2F6 in the presence and absence of HA-RYBP and immunoprecipitated with the indicated antibodies. Non-specific bands are indicated by asterisks.

Figure 4 E2F6 is a component of the mammalian polycomb complex (A) C-33A cells were transiently transfected with expression vectors encoding E2F6 and/or HA-Ring1A, labeled with [^{35}S]methionine and immunoprecipitated with the indicated antibodies. (B) ML-1 cell lysates were immunoprecipitated with either control (pre-immune serum) or anti-Ring1 antibodies (ASA8), resolved by SDS-PAGE and immunoblotted with an anti E2F-6 (LLF6-2) antibody (left panel). Additionally, ML-1 lysates were immunoprecipitated with control (12CA5), anti-E2F6 (LLF6-1) or anti-RYBP (LLRYBP-1) antibodies, resolved by SDS-PAGE and immunoblotted with the anti Ring1 (ASA8) antibody (right panel). The immunoglobulin heavy chain band is denoted by an asterisk. (C) C33-A cells were transfected as in (A) with either E2F6 or HA-Bmi1 and immunoprecipitated with the indicated antibodies. The data shown is from the same exposure of a single gel. We consistently immunoprecipitated a higher level of E2F6 protein in the presence rather than the absence of Bmi1. This reflects a comparable increase in the E2F6 protein levels (as judged by western blotting of whole cell lysates) that occurs when it is co-expressed with Bmi1. (D) ML-1 cells were immunoprecipitated as in (B) with either normal rabbit serum, anti-MEL-18, anti-mph1 or anti-Bmi1antibodies and immunoblotted with anti-E2F6 (LLF6-2) or anti-Bmi1 antibodies.

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Chapter 4

Investigating the biological properties of the E2F6 containing polycomb complex

Jeffrey M. Trimarchi, Jessica Wen and Jacqueline Lees

Author's contribution: Figures 1, 2, 3, and 4

Abstract

E2F6 is the most recently identified member of the E2F family of transcription factors. It is only distantly related to the other E2Fs and E2F6 lacks the sequences that mediate transactivation and pocket protein binding. Consistent with its structure, E2F6 can behave as a repressor of transcription *in vitro*. In an effort to understand this repression, we have previously isolated RYBP, a member of the mammalian PcG complex, as a specific E2F6 interacting protein. Furthermore, E2F6 can associate with several polycomb proteins, including the oncogene Bmi1. Here we begin to characterize the biological consequences of these interactions. We show that these proteins have similar expression profiles across the cell cycle. Additionally, overexpression of E2F6 can decrease $p19^{ARF}$ expression in a similar manner to Bmi1. Finally, we demonstrate that overexpression of RYBP can lead to the formation of condensed chromatin. These findings suggest that the E2F6-containing PcG complex may influence the expression of cell cycle related genes through a modification of the local chromatin environment.

Introduction

The E2F family of transcriptional factors plays a critical role in the control of cellular proliferation and differentiation (Dyson 1998). They act by regulating the activity of a set of genes whose expression is essential for cell cycle progression and DNA synthesis. To date, eight genes have been cloned that encode components of the E2F transcriptional activity (Dyson 1998). Their protein products can be divided into two groups, the E2Fs (1-6) and the DPs (1, 2), that form heterodimers to generate functional E2F complexes (Helin et al. 1993; Krek et al. 1993; Wu et al. 1995; Chapter 2). Although dimerization with the DP protein is necessary for activity, it is the E2F moiety that confers the functional specificity on E2F/DP complexes (Dyson 1998).

The E2F group can be divided into three distinct subclasses based upon sequence homology and functional properties. E2F1, E2F2, and E2F3 represent the first subclass. These "activating E2Fs", when bound to DP, have high transcriptional activity and are sufficient to drive quiescent cells into S phase (Kowalik et al. 1995; DeGregori et al. 1997). In normal cells, the "activating E2Fs" are controlled by their association with the retinoblastoma protein (pRB), the first identified tumor suppressor (Hiebert et al. 1992).

E2F4 and E2F5 represent the second subclass. Unlike the "activating E2Fs", E2F4 and E2F5 are poor transcriptional activators, are unable to induce quiescent cells to re-enter the cell cycle, and are believed to play an important role in the repression of E2F responsive genes (Lukas et al. 1996; Muller et al. 1997; Verona et al. 1997).

E2F6, the most recently identified E2F family member, represents a third subclass of the E2F family. While the central portion of the E2F6 protein shares significant

homology with the sequences in E2Fs1-5 that mediate their DP dimerization and DNA binding properties, there is a high degree of sequence divergence outside of these domains (Chapter 2). First, the N-terminus of E2F6 bears no similarity to those of the other E2F family members. Second, and most importantly, E2F6 terminates 42 amino acids after the DP dimerization domain and, therefore, lacks the sequences that mediate the transcriptional activation and pRB binding properties of the other E2Fs.

Using a yeast-two hybrid assay, we identified RYBP (Ring1 and YY1 binding protein), a recently isolated member of the mammalian polycomb group of proteins (PcG), as an E2F6 interacting partner. Despite the fact that the RYBP-interacting domain in E2F6 is highly conserved among all the members of the E2F family, the RYBP interaction was a specific property of E2F6. Consistent with these observations, we demonstrated an association between endogenous E2F6 and additional PcG proteins, including Ring1a, MEL-18, mph1 and Bmi1. These data indicated that the ability of E2F6 to behave as a transcriptional repressor could depend upon its ability to recruit the PcG complex.

Originally identified in *Drosophila melanogaster*, the PcG proteins are essential for maintaining the transcriptional repression of genes important for development (reviewed in (Kennison 1995). In PcG mutant *Drosophila* embryos, the initial patterns of homeotic (Hox) gene expression are established correctly early in development, but are not subsequently maintained. The PcG proteins are believed to function through the formation of large multisubunit complexes, arising through mutual interaction among highly conserved protein motifs (Kyba and Brock 1998; Shao et al. 1999; Saurin et al. 2001). Promoter mapping studies have identified DNA sequences, called polycomb

response elements (PREs), which are sufficient to mediate the silencing effect of the PcG proteins. However, these fragments are quite large (100-300 bp) and the exact mechanism by which the PcG complex interacts with them is not well understood (Strutt and Paro 1997).

Many mammalian PcG homologues have been identified by virtue of their homology with the *Drosophila* proteins or as a result of yeast-two-hybrid screens reviewed in (Gould 1997; Satijn and Otte 1999; Bardos et al. 2000). These proteins are also thought to silence genes in a multisubunit complex (Alkema et al. 1997). Unlike the case in *Drosophila*, however, no DNA sequences have been identified that can confer PcG mediated repression. In fact only a handful of potential DNA binding proteins (MEL-18, YY1, E2F6) have been found to associate with the mammalian PcG complex. It is still unclear whether any of these factors is actually responsible for targeting the PcG complex to the DNA.

Many mammalian PcG genes have been shown to play a key role in the regulation of cellular proliferation. Bmi1, for example, was originally identified through its ability to cooperate with an *Eμ-myc* transgene in the generation of murine leukemias (Haupt et al. 1991; van Lohuizen et al. 1991). The analysis of Bmi1 deficient mice and cells has revealed that loss of Bmi1 results in the upregulation of two tumor suppressor genes, $p19^{ARF}$ and $p16^{INK4a}$ (Jacobs et al. 1999a; Jacobs et al. 1999b). Although it has been inferred that Bmi1 directly mediates the transcriptional repression of these genes, this has yet to be demonstrated. Additionally, the mechanism by which the Bmi1-containing PcG complex is recruited to its target genes is currently unknown.

Given our previous finding that E2F6 and Bmi1 can associate in the same PcG complex, we wished to explore the consequences of this interaction further. In this study, we find that E2F6 and its PcG interacting partners have a similar cell cycle expression pattern. Additionally, overexpression of E2F6 can influence the expression of $p19^{ARF}$ in a similar manner to Bmi1. Furthermore, RYBP, an E2F6 interacting protein, appears to be capable of inducing chromosome condensation. These data reveal some potential biological functions for the E2F6/RYBP/Bmi1 PcG complex.

Materials and Methods

Antibody production Full-length His_6-tagged murine Bmi1 (amino acids 1-326) protein was expressed in bacteria, purified over a Ni^{2+}-nitrilotriacetic acid-agarose resin (Qiagen) and used to immunize Balb/c mice. The resulting polyclonal antiserum was monitored for its ability to recognize overexpressed Bmi1 in Western blotting, immunoprecipitation and immunofluorescence assays. Mice generating Bmi1 antibodies were sacrificed and their spleens removed. The isolated splenocytes were fused to SP2/O cells by a polyethylene glycol-based method. After 10 days, the supernatants from the resulting hybridoma cell lines were screen for their ability to recognize recombinant Bmi1 in an ELISA. The positive cell lines were single-cell cloned and re-tested for Bmi1 reactivity by Western blotting, immunoprecipitation and immunofluorescence.

T cell isolation and western blotting To obtain a population of primary human T cells, buffy coats of human blood were purchased from the Massachusetts General Hospital Blood Bank. The isolation of the T cells and subsequent stimulation were performed as described (Moberg et al. 1996). Briefly, the mononuclear cell layer was isolated by centrifugation on a Ficoll-Paque cushion (Pharmacia) and then washed extensively with phosphate-buffered saline (PBS). The cells were resuspended in RPMI supplemented with 10% fetal calf serum (FCS) and stimulated to proliferate by the addition of 1 μg/ml of phytohemagglutinin (Murex, UK). Cell pellets were harvested every 6 hours for a total of 60 hours. At each time point, cell cycle progression was monitored by the incorporation of [^{3}H]thymidine. One milliliter of T cells was incubated with 10 μCi of

[^{3}H]thymidine for 30 minutes. After washing in PBS, these cells were lysed in 0.3N NaOH, spotted onto GF/C glass filters (Whatman), precipitated in trichloroacetic acid and counted in a scintillation counter.

Whole cell extracts were prepared and Western blotting performed as previously described (Moberg et al. 1996). Anti-E2F3 (LLF3-1), anti-E2F6 (LLF6-2), anti-RYBP (LLRYBP-2), and anti-Ring1a (ASA8) antibodies have been described elsewhere (Moberg et al. 1996; Saurin et al. 1998; Chapter 3).

Plasmid construction Full-length E2F6 was excised from pCMV-E2F6 by a BamHI digestion and subcloned into the pBABE retroviral vector (Morgenstern and Land 1990). The plasmids pCMV-E2F3, pCMV-E2F6, pCMV-RYBP, pHACMV-RYBP, pCMVHA-YAF2, pHACMV-Ring1a, and pHA-Bmi1 have been described (Moberg et al. 1996; Kalenik et al. 1997; Chapter 3).

Retroviral-mediated gene transfer Mouse embryonic fibroblasts (MEFs) were prepared from E13.5 embryos derived from wildtype mice. After removing the head and internal organs, the embryos were minced and incubated in trypsin for 45 minutes at 37°C. The cells were resuspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS, 50 U/ml penicillin and 50 μg/ml streptomycin.

The ecotropic retrovirus packaging cell line Phoenix (Gary Nolan) was plated at a density of $5x10^6$ cells/10 cm plate. Twenty-four hours later, the media was removed and replaced with DMEM supplemented with 10% FCS and 25 μM chloroquinone (Sigma). These cells were transiently transfected with 20μg of retroviral plasmid using the calcium

phosphate precipitate method. After 10 hours at 37°C the media was removed, replaced with DMEM supplemented with 10% FCS and the cells incubated for 24 hours. The supernatant was supplemented with 4 μg/ml polybrene and 1 ml of fresh FCS, filtered through a 10 cc syringe/2 μM filter and used to infect two plates of MEFs ($7.5x10^5$ cells/10 cm plate). Fresh media was added to the Phoenix cells and these steps were repeated for a second infection. The infected MEFs were selected in puromycin (2 μg/ml) for four days. Whole cell extracts were Western blotted with the indicated antibodies [anti-E2F6 (LLF6-2), anti-p27 (Santa Cruz, sc-528), $p19^{ARF}$ (Novus Biologicals, NB 200-106)].

Immunofluorescence U2OS cells were plated on glass cover slips and grown to a confluency of 50%. These cells were transiently transfected with pHACMV-RYBP, pHACMV-YAF2 or pHACMV-neo-Bam vectors by calcium phosphate precipitation. After transfection, the cells were fixed in 4% paraformaldehyde for 15 min. and the cell membranes permeabilized with methanol:acetone (50:50) for 2 min. After washing in PBS, the cells were incubated for 30 min. at 37°C with a 1:25 dilution of the anti-HA tag monoclonal antibody (12CA5). Cells were washed again in PBS and incubated for 30 min. at 37°C with a secondary antibody [fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody at a 1:1,000 dilution (Cappel)]. The cover slips were then washed with PBS, incubated with 4'6-diamidino-2-phenylindole (DAPI, 0.1 mg/ml) for 5 min., washed again, and mounted onto glass slides with Fluoromount G (EMS).

Results

Cell cycle regulation of the E2F6-containing PcG complex We have recently demonstrated that E2F6 can associate with several members of the mammalian polycomb complex, including the oncogene Bmi1 (Chapter 3). To begin to address the biological role of the E2F6-associated polycomb complex, we examined the expression patterns of E2F6 and its interacting partners across the cell cycle. Primary human T lymphocytes were isolated from peripheral blood over a Ficoll-Paque cushion and stimulated to enter the cell cycle with phytohemagglutinin (PHA). Time points were collected every 6 hours for a total of 60 hours. At each time point, the amount of DNA synthesis was monitored by measuring the uptake of [^{3}H]thymidine. In this experiment, the peak of S phase occurred at 42 hours, indicating that most of the cell population traversed the G1/S transition shortly before this time (Fig. 1A).

The expression pattern for E2F6 and its associated proteins was determined by western blot analysis of whole-cell lysates generated for each time point. As a control, we examined the expression of E2F3a, a protein known to be induced just before the G1/S transition. Consistent with previous studies (Moberg et al. 1996), E2F3a was not detected until 36 hours after PHA stimulation, just 6 hours prior to the initiation of S phase (Fig.1B top panel). RYBP followed a similar pattern as E2F3a as it was undetectable 6 hours prior to the onset of DNA synthesis (Fig. 1B third panel). E2F6, however, began to be detectable just 12 hours after PHA addition, peaked at 36 hours, and declined over the remainder of the time course (Fig. 1B second panel). Similarly, Ring1a and Bmi1 protein was clearly observed by 12 hours after stimulation, peaked

shortly thereafter and declined near the end of the timecourse (Fig. 1B fourth and fifth panels). Thus, E2F6, RYBP, Ring1a and Bmi1 all display peak expression immediately before DNA replication.

E2F6, the PcG, and senescence Overexpression of the Bmi1 protein results in the downregulation of the $p19^{ARF}$ tumor suppressor and a concomitant bypass of the normal cellular senescence process (Jacobs et al. 1999a). Given that E2F6 can associate with the Bmi1-containing polycomb complex, we sought to establish whether E2F6 could play a similar role in the regulation of $p19^{ARF}$ and cellular senescence. To determine if overexpression of E2F6 could influence the levels of $p19^{ARF}$ in a similar manner to Bmi1, we constructed an E2F6 retroviral vector (pBABE-E2F6). Phoenix packaging cells were transiently transfected with either pBABE or pBABE-E2F6 and the resulting supernatants were harvested. These supernatants, containing the recombinant replication-deficient retroviruses, were filtered and used to infect mouse embryonic fibroblasts (MEFs). The pBABE retroviral vector coexpresses a puromycin phosphotransferase gene allowing for selection of pure populations in four days. At the end of the puromycin selection process, the MEFs infected with the pBABE-E2F6 producing retrovirus were observed to express E2F6 at higher levels than the control infected population (Fig. 2A left panel).

When these E2F6-expressing cells were western blotted for $p19^{ARF}$, they were found to express slightly decreased levels of $p19^{ARF}$ protein as compared to cells infected with a control retrovirus (Fig 2A middle panel). This was not merely due to a general reduction in all cellular protein levels, since cells infected with either control or E2F6-producing retroviruses expressed normal levels of p27 (Fig. 2A right panel). Unfortunately, a direct

comparison with Bmi1-expressing retroviruses was not possible since, in our hands, Bmi1 overexpression in MEFs was not observed with a host of different retroviral vectors. Nevertheless, the results obtained with the E2F6 retrovirus suggest that, either directly or indirectly, overexpression of E2F6 can downregulate the expression of $p19^{ARF}$.

When MEFs are passaged in culture the cells eventually become larger, flatten out on the dish, and cease proliferating. Accompanying these changes in cell morphology are changes in gene expression. For example, when MEFS were serially transferred according to a 3T9 protocol, $p19^{ARF}$ protein was found to be induced at early passages (passage 5) and to increase steadily thereafter (Zindy et al. 1998). This accumulation of $p19^{ARF}$ protein was shown to correlate with the decrease in cellular proliferation as the MEFs underwent senescence. Since both Bmi1 (Jacobs et al. 1999a) and E2F6 have been shown to influence the expression of $p19^{ARF}$, we compared the levels of E2F6, its associated polycomb members, and $p19^{ARF}$ in MEFs that had been serially passaged along a 3T3 protocol. Unfortunately, we could not examine the levels of Bmi1 protein in this experiment due to the fact that our antibody did not specifically recognize the endogenous Bmi1 protein (data not shown). In our experiment, $p19^{ARF}$ protein levels did increase (Fig. 2B) and this upregulation paralleled a decrease in cellular proliferation (data not shown). In fact, $p19^{ARF}$ levels diminished during the last time points harvested and this correlated with cells escaping the senescence program and becoming 3T3 cells. During the entire time course, however, the levels of E2F6 and RYBP remained constant (Fig. 2B). Although we cannot rule out the possibility that E2F6 and RYBP were post-translationally modified differentially across the time course, we did not detect a

significant change in their expression levels that could account for the changes in $p19^{ARF}$ expression.

Subcellular localization of E2F6/RYBP and chromosome condensation In U2OS cells, several PcG proteins, including Ring1a and Bmi1, have been found to localize in large nuclear "speckle-like" structures called PcG bodies (Schoorlemmer et al. 1997; Saurin et al. 1998). However, both endogenous and overexpressed RYBP was observed throughout the nucleus (Garcia et al. 1999). Given that E2F6 associates with several members of the PcG complex, we wished to determine its subcellular localization. U2OS cells were transiently transfected with an expression vector encoding E2F6 and processed for indirect immunofluorescence. Using multiple different E2F6 specific monoclonal antibodies, we found that overexpressed E2F6 localizes in a diffuse nuclear pattern (data not shown). In fact, the endogenous E2F protein was distributed throughout the nucleus as well (data not shown). Thus, the subcellular localization of E2F6 is more similar to that reported for RYBP than other PcG proteins.

In the course of these experiments, we observed a surprising phenomenon in U2OS cells transiently transfected with an expression construct for RYBP. Staining of the transfected cells with DAPI revealed that the DNA in many of the RYBP-positive cells (40%, 74%, and 57% for RYBP-positive cells versus 0%, 0%, and 2% respectively for the controls in three independent transfections) had become condensed into a series of "DNA blobs" (Fig. 3A). This chromosome condensation did not seem to result from apoptosis as the "DNA blobs" were not TUNEL positive (data not shown) and the dying cells were clearly distinct (Fig. 3A white arrow) from the other RYBP-positive cells. In

addition, this phenomenon was not merely a consequence of the cells being driven in mitosis, as these RYBP-positive cells did not co-stain with an anti-phosphoserine H3 antibody, an established mitosis marker (data not shown). Finally, the highly related protein, YAF2, (Kalenik et al. 1997) failed to induce these "DNA blobs" when similarly transfected into U2OS cells (Fig. 3B). Thus, overexpression of RYBP specifically induces the formation of DNA structures reminiscent of condensed chromatin.

Since we observed some variation in the percentage of RYBP-transfected cells that showed these "DNA blobs", we wished to more fully characterize this phenomenon. Perhaps the variation in this phenomenon was a result of the "DNA blobs" only being a temporary and unstable structure. To test this hypothesis, we transiently transfected U2OS cells with an RYBP expression vector, harvested cells at 0, 6, 18, 36, and 48 hours after transfection, and processed these cover slips for immunofluorescence. Representative fields from the 6, 18 and 36 hour time points are shown in figure 4. We found that the appearance of these "DNA blob" structures is in fact highly transient. None of the RYBP-transfected cells showed this phenomenon at the 0 and 6 hour timepoints. Eighteen hours after transfection, 36% of the RYBP-positive cells had condensed their DNA (for example Fig. 4B). By 36 hours, however, these structures had disappeared, although we still observed RYBP-positive cells (see Fig. 4C). Although we do not know the fate of any individual cell that displays the "DNA blob" structures, this condensed chromatin phenomenon does not persist in RYBP overexpressing cells.

Discussion

E2F6, the most recently cloned member of the E2F family, is only distantly related to the other E2Fs. In particular, E2F6 lacks the sequences required for pRB binding or transactivation and it can behave as a transcriptional repressor *in vitro* (Gaubatz et al. 1998; Chapter 2). Consistent with the ability of E2F6 to repress transcription, we identified RYBP, a member of the mammalian polycomb repressor complex, as a specific E2F6-interacting protein. In addition, the endogenous E2F6 protein is associated with other PcG proteins, including Ring1a, MEL-18, mph1 and the oncoprotein Bmi1 (Chapter 3). In this study, we extend these results by showing that E2F6, RYBP, Ring1a and Bmi1 are expressed in a similar manner across the cell cycle. Additionally, overexpression of E2F6 can reduce the expression of the tumor suppressor protein $p19^{ARF}$. Finally, we present evidence that RYBP may be involved in the formation of condensed chromatin.

Since E2F6 is homologous to the other E2Fs, especially in the DNA binding domain, our observations that E2F6 can associate with the PcG complex raise the possibility that E2F6 might play a role in the targeting of polycomb proteins to DNA. Despite extensive efforts to elucidate the DNA binding mechanisms of the *Drosphila* PcG complexes, the few DNA binding factors found, thus far, in association with known PcG proteins cannot account for all the PcG DNA binding activity (Strutt and Paro 1997; Brown et al. 1998).

While numerous mammalian PcG homologues have been identified, very little is known as well about the mechanisms by which these complexes target DNA and which genes are targeted. Mutant mouse models have confirmed that the PcG complex

regulates the mammalian Hox genes in an analogous manner to that in *Drosophila* (van der Lugt et al. 1994; Akasaka et al. 1996; Core et al. 1997) (del Mar Lorente et al. 2000). The analysis of *Bmi1*$^{-/-}$ mice and MEFs has revealed that p19^{ARF} is a novel mammalian PcG target gene (Jacobs et al. 1999a; Jacobs et al. 1999b). Loss of Bmi1 results in the derepression of p19^{ARF} and an induction of premature cellular senescence (Jacobs et al. 1999a). Furthermore, infection of MEFs with a Bmi1-expressing retrovirus resulted in a 2.4 fold reduction in p19^{ARF} transcript levels (Jacobs et al. 1999a). In the present study, we demonstrate that MEFs infected with a retrovirus encoding E2F6 express less p19^{ARF} than control MEFs. Unfortunately, in our hands, we could not achieve Bmi1 overexpression in MEFs using several different retroviral vectors. Whatever the reason for the inability of our MEFs to tolerate high levels of Bmi1, we could not directly compare the effects of E2F6 with those of Bmi1. Nevertheless, the fact that E2F6 can influence p19ARF expression in a similar manner to Bmi1, coupled with the demonstration that these two proteins can associate, strongly suggests that they function as part of a complex *in vivo*.

While the mechanism by which the mammalian PcG complex recognizes the DNA is still unclear, it has been widely proposed that PcG complex exerts its repressive effects through modifying the structure of chromatin. The Polycomb repressive complex 1 (PRC1) isolated from *Drosophila* embryos was able to prevent nucleosome remodeling when pre-incubated with the DNA (Shao et al. 1999). Furthermore, the chromodomain motif present in the heterochromatin associated protein (HP1) was recently shown to bind to methylated histones. It is through this binding that HP1 is believed to facilitate heterochromatin formation and spread its silencing effect along the chromosome

(Bannister et al. 2001). The PcG protein M33 also possesses a N-terminal chromodomain that can bind to methylated histones (Lachner et al. 2001). Therefore, by analogy with HP1, M33 may function to target mammalian PcG complexes to the DNA via histone modifications and affect the local chromatin structure.

Here we have shown that overexpression of another PcG protein, RYBP, can lead to the formation of condensed "DNA blob" structures. Phenomena known to involve condensed chromatin such as apoptosis and mitosis did not appear to explain these results. However, several recent reports examining the consequences of overexpression of HP1 in mammalian cells revealed numerous similarities with the RYBP-induced phenomenon. First, overexpressed HP1α, one mammalian HP1 isoform, localized to regions of bright Hoechst staining (blob-like structures) known to be areas rich in heterochromatin (Nielsen et al. 2001). Second, microinjection of HP1α protein into mammalian cells revealed that in the first few hours post-injection, HP1α was concentrated along the periphery of the nucleus (Kourmouli et al. 2000). Similarly, transfected RYBP also appeared to be more abundant along the edge of the nucleus (see Fig. 4C). These results suggest that RYBP, either directly or indirectly, can influence the formation of heterochromatin much like HP1. This finding indicates that through RYBP, the E2F6-containing PcG complex may repress transcription by influencing the local chromatin environment.

Acknowledgements

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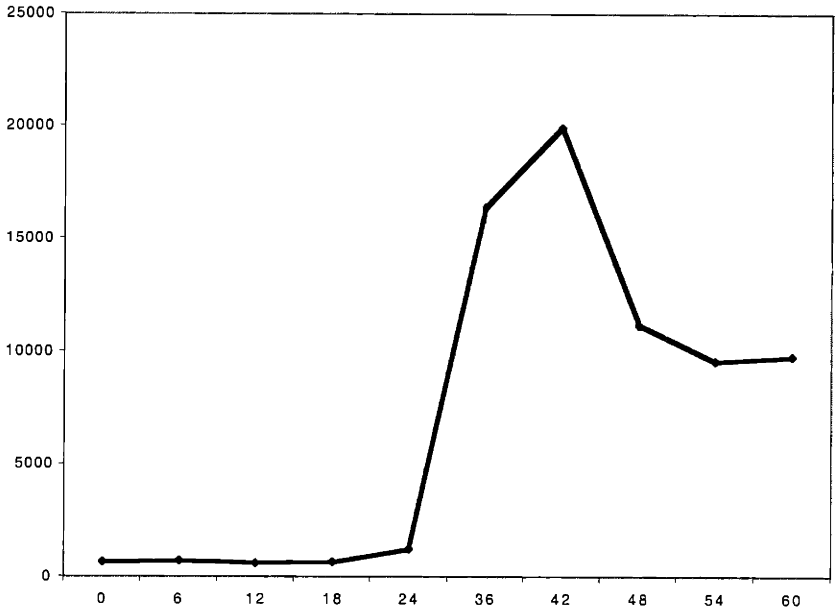
Figure 1

A.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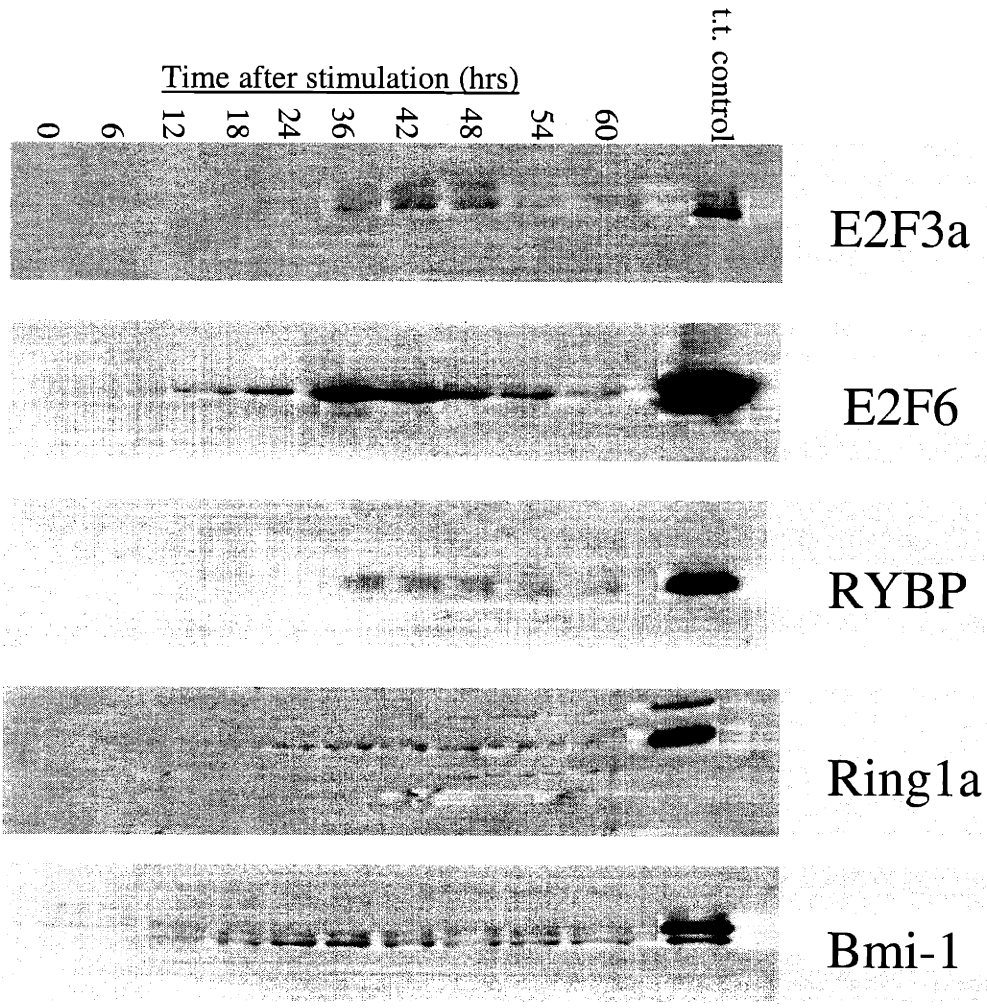
B.


Time after stimulation (hrs)
0
6
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18
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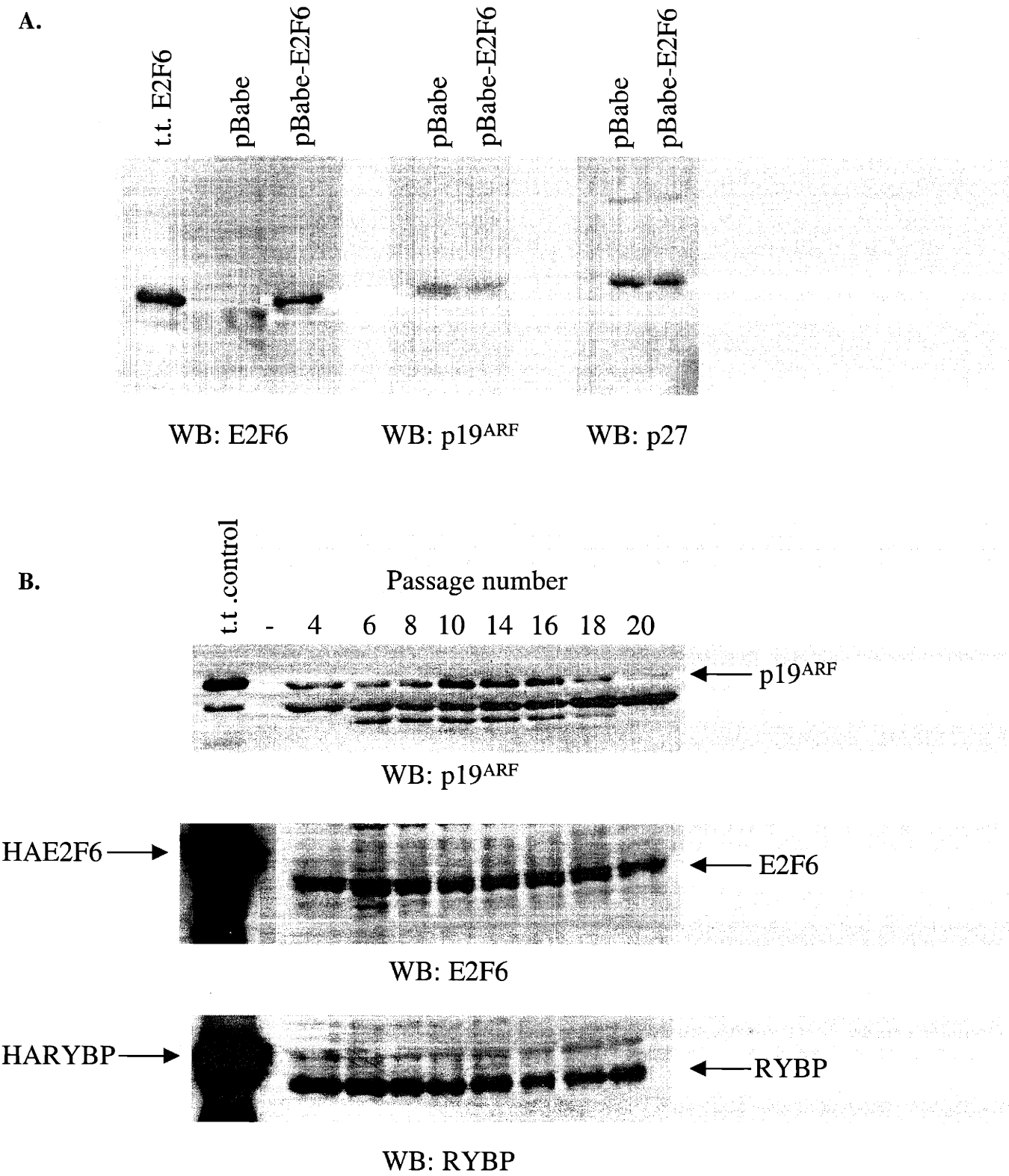
E2F3a
E2F6
RYBP
Ring1a
Bmi-1

Figure 2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WB: p19ARF
WB: p27
B.
t.t .control
Passage number
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Figure 3

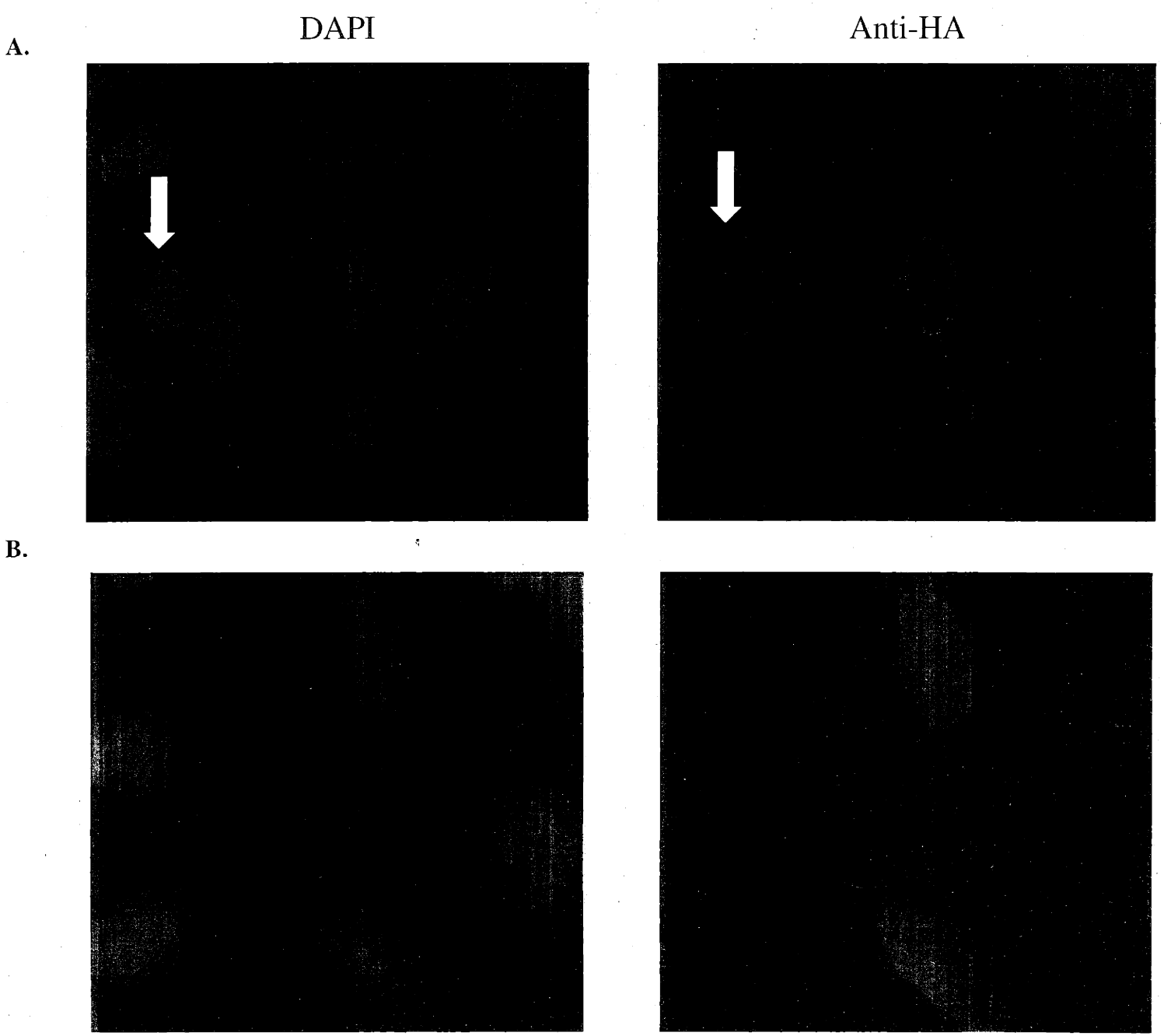

Figure 4

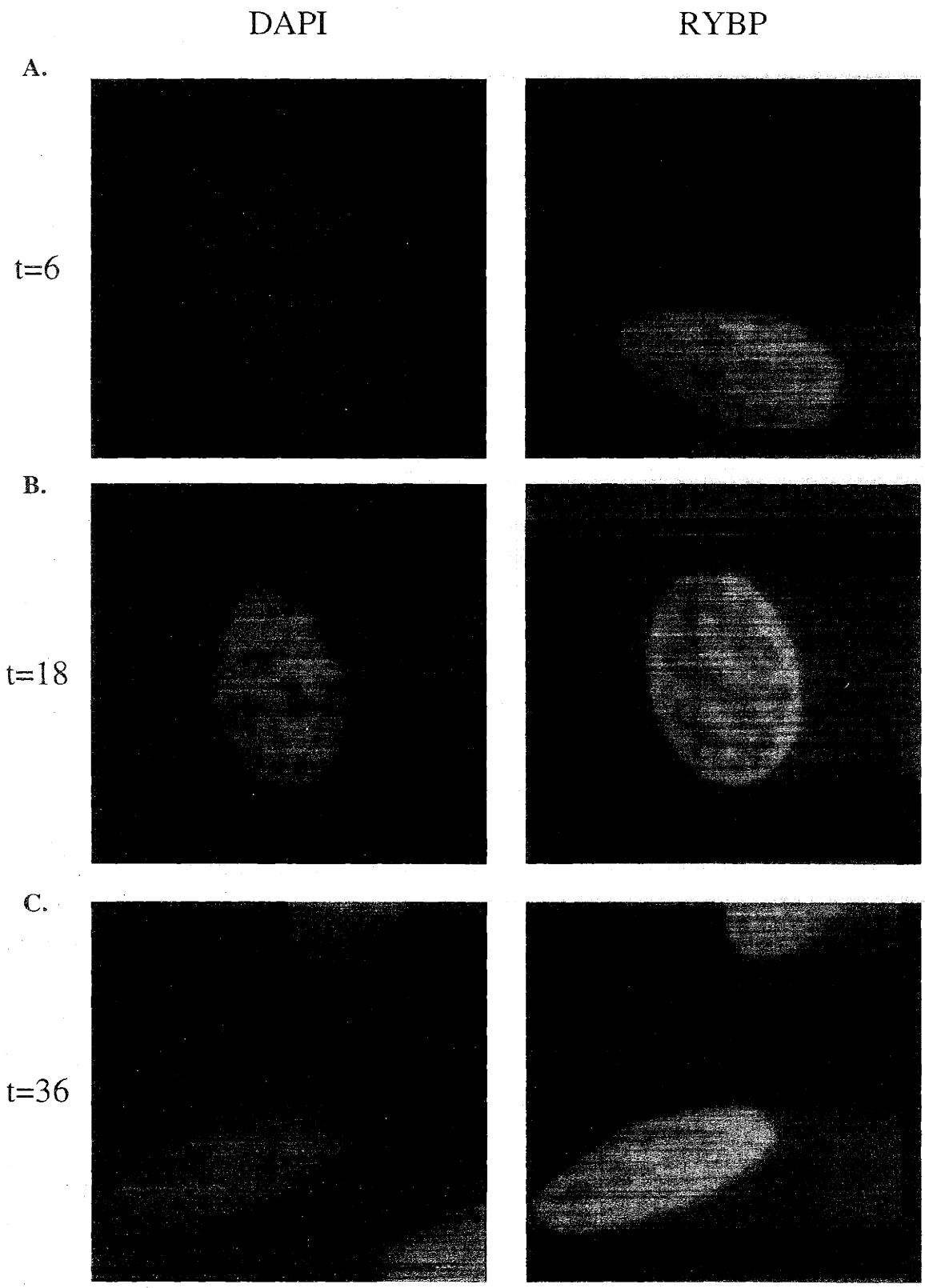

Figure Legends

Fig. 1 Cell cycle expression patterns of E2F6 and its associated PcG proteins. (A) [^{3}H]thymidine incorporation into primary human T cells at the indicated time after stimulation with PHA. (B) Western blot analysis of E2F3a, E2F6, RYBP, Ring1a and Bmi1 from T cell extracts (50 μg per lane). The membranes were blotted with anti-E2F3 (LLF3-1), anti-E2F6 (LLF6-2), anti-RYBP (LLRYBP-2), Ring1a (ASA8) or Bmi1 (LLBMI1-1) antibodies.

Fig. 2 Effects of E2F6 on $p19^{ARF}$ expression. (A) Phoenix cells were transiently transfected with pBABE-puro or pBABE-E2F6-puro vectors. The media was harvested and used to infect mouse embryonic fibroblasts (MEFs). The infected MEFs were selected in puromycin (2 μg/ml) for four days. Whole cell extracts were Western blotted with the indicated antibodies [anti-E2F6 (LLF6-2), anti-p27 (Santa Cruz, sc-528), $p19^{ARF}$ (Novus Biologicals, NB 200-106)]. (B) MEFs were plated at $3X10^5$ cells/6 cm plate. When they reached confluence, the cells were counted and re-plated at $3X10^5$ cells/plate. At the passages indicated whole cell extracts (100 μg) were Western blotted with anti-$p19^{ARF}$ (Novus Biologicals, NB 200-106), anti-E2F6 (LLF6-2), or anti-RYBP (LLRYBP-2). Extract from $p53^{-/-}$ MEFs was used as a control for $p19^{ARF}$.

Fig. 3 Exogenous RYBP induces "DNA blobs". (A) U2OS cells were plated on coverslips and transiently transfected with a pCMV-RYBP expression construct. The DNA was stained with DAPI (left panel). Immunofluorescence of RYBP was detected

using an anti-HA tag antibody (12CA5) and a FITC-conjugated goat anti-mouse secondary antibody (right panel). The white arrow indicates an apoptotic cell. (B) U2OS cells were plated as in (A) and transiently transfected with a pHACMV-YAF2 construct. The cells were either stained with DAPI (left panel) or an anti-HA tag antibody (12CA5) to detect HA-YAF2 (right panel).

Fig. 4 Effects of exogenous RYBP at different time points. U2OS cells were plated on coverslips, transiently transfected with a pCMV-RYBP expression vector, and processed for indirect immunofluorescence at 6 (A), 18 (B), and 36 (C) hours after transfection.

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Chapter 5

Conclusions

This work details the identification and characterization of E2F6, a novel member of the E2F transcriptional factor family. In Chapter 2, we describe the initial cloning of E2F6 and demonstrate that this protein has similar heterodimerization and DNA binding properties as the other E2Fs. However, instead of activating transcription, E2F6 behaved as a repressor. To begin to understand the mechanism behind this repression, we screened for E2F6 interacting proteins in Chapter 3. We found that E2F6 could interact specifically with RYBP, a recently identified component of the mammalian polycomb complex. In addition, E2F6 could associate with several other members of the polycomb group (PcG). In Chapter 4, we began to examine the role that this complex might play in the control of cellular proliferation. Together these findings raise a number of questions as to what role E2F6 plays in the DNA binding activity of the polycomb complex and what role the PcG complex plays in E2F6-mediated repression.

A. E2F6 as an E2F family member

E2F6 was originally cloned through the properties it has in common with the other E2F family members. We (Chapter 2) and others (Cartwright et al. 1998; Gaubatz et al. 1998) identified E2F6 by virtue of its high degree of homology with the E2F proteins. This homology is most pronounced in the DNA binding domain (50-60% identical) and the dimerization domain (~30% identical). Consistent with these data, E2F6 can bind to DP1 and DP2, in transfection experiments and at endogenous protein levels (Chapter 2; Morkel et al. 1997; Cartwright et al. 1998; Gaubatz et al. 1998 and data not shown). Additionally, E2F6/DP complexes can bind to DNA in a sequence specific manner.

These results strongly suggested that E2F6 was a member of the E2F family of transcription factors.

Despite the dimerization and DNA binding results, even in the initial characterization, E2F6 possessed properties distinct from the other E2Fs. At a significant proportion of the conserved residues, E2F6 had alternative codon usage from that found in the other E2Fs. In addition, residues known to distinguish the "activating E2Fs" from the "repressive E2Fs" were often completely different in E2F6. Outside of the conserved domains, the sequence of E2F6 was even more distinct from the rest of the E2F family. The N-terminus was of an intermediate length and resembled neither subclass of the E2F family. Most strikingly, the C-terminus of E2F6 truncates 42 amino acids after the dimerization domain. E2F6, therefore, lacks the sequences that mediate both pocket protein binding and transactivation in the other E2Fs. Taken together, these data indicate that E2F6 represents a new class of the E2F family.

The functional properties displayed by E2F6 also distinguish it from the other E2F proteins. Consistent with the sequence information, E2F6 failed to bind any of the known pocket proteins and it did not activate an E2F-responsive reporter gene. Furthermore, while E2F6/DP complexes were competent to bind E2F DNA binding sites, we consistently observed lower E2F DNA binding activity in extracts derived from cells transfected with E2F6 rather than one of the other E2Fs (Chapter 2). This observation held true for several different E2F consensus DNA binding sites (Chapter 2), including one that was reported to be a preferred E2F6 binding site (Morkel et al. 1997). The crystal structure of a portion of E2F4/DP2 bound to DNA has been solved and the critical residues for DNA binding identified (Zheng et al. 1999). A comparison with E2F6

revealed that all of the residues shown to contact the DNA in the crystal structure are present in E2F6 as well. Therefore, there must be some other intrinsic difference in the E2F6/DP complexes that explains their reduced DNA binding capability.

One potential explanation for the reduced DNA binding affinity of E2F6/DP complexes is that they may require an additional factor for high affinity DNA binding. Along these lines, we have tested the ability of E2F6/RYBP and E2F6/RYBP/DP complexes to bind to consensus E2F sites. E2F6/RYBP complexes failed to bind E2F-DNA binding sites and the presence of RYBP did not significantly alter the low affinity DNA binding activity of E2F6/DP complexes (our unpublished data). Significantly, although we possess a number of monoclonal antibodies that supershift E2F6/DP complexes *in vitro*, we have been unable to detect endogenous E2F6/DP DNA binding activity (data not shown). These results raise clear questions about the DNA binding properties of E2F6-containing complexes *in vivo*. At this point, it is possible that E2F6/DP complexes require other factor(s) for high affinity DNA binding or that E2F6/DP/Factor X complexes bind to a non-E2F DNA binding site. A further complication comes from our inability to find DP in RYBP immunoprecipitates (see below). Additionally, we have been unable to detect DP in immunoprecipitates from any of the PcG antibodies currently available to us (see Chapter 3 and data not shown). In conclusion, E2F6, despite its similarities to the other E2Fs, appears to function very differently from the rest of the E2F family.

B. E2F6 and the PcG complex

To begin to understand the distinct properties of E2F6, we screened for interacting proteins. We discovered that E2F6 was associated with several known members of the mammalian polycomb complex, including RYBP, Ring1a, MEL-18, mph1, and Bmi1 (Chapter 3). Since E2F6 can behave as a transcriptional repressor (Gaubatz et al. 1998) and the mammalian PcG complex plays an important role in transcriptional repression, a link between the two provides insight into how mammalian PcG proteins target the DNA and how E2F6 can inhibit transactivation.

(i) Composition of mammalian PcG complexes

Studies in *Drosophila* and mice have revealed the existence of at least two distinct polycomb complexes, one acting early in development (Eed/Esc containing) and the other acting later in development (Bmi1/Pc containing) (reviewed in Satijn and Otte 1999a). As additional PcG proteins have been identified, each factor has been shown to interact with a single complex, leading to the idea that these two complexes are completely distinct. E2F6 has been found in association with the RYBP/Ring1a/Bmi1-containing complex (Chapter 3), while the transcription factor YY1 has been shown to interact specifically with the Eed-containing PcG complex (Satijn et al. 2001). However, our subsequent data (Appendix B) demonstrates that E2F6 can associate with YY1 in cell extracts and Ring1a and YY1 can interact robustly in a co-transfection assay. Furthermore, in our experiments RYBP and YY1 fail to interact when transiently transfected into mammalian cells. This result is in direct contrast to another report that observed a direct interaction between RYBP and YY1 *in vitro*. The reasons for these discrepancies are still unclear, but each of these studies used different assays and PcG proteins from distinct organisms. Taken together, our analysis of E2F6 casts some doubt

upon the simple model of two completely distinct mammalian PcG complexes. It appears, in fact, that either the two complexes can share common components or that there is a physical interaction between the Bmi1 and Eed containing PcG complexes.

Even within the Bmi1-containing PcG complexes, there is a strong likelihood that there are several variant complexes, each of which contains a different subset of factors. First, in mammals, there are instances where two or more homologues exist to a single *Drosophila* PcG protein. For example, there are at least three Pc homologues (M33, hPc2, hPc3), 2 ph homologues (HPH1, HPH2), and 2 Psc homologues (Bmi1 and Mel-18) present in mammalian cells. Knockout mouse models suggest that these proteins play partially overlapping roles in the regulation of Hox genes. However, each of these deficient mice displays some unique phenotypes, suggesting that these homologues perform some distinct or tissue specific functions as well. Our data shows that E2F6 can associate with several of these PcG proteins. In some cases, we find E2F6 can interact with both related homologues (Bmi1 and Mel-18, RYBP and YAF2). It is still unknown, however, whether these interactions all occur in the context of the same complex or if E2F6 participates in multiple different "late" PcG complexes.

Additionally, biochemical purification of the *Drosophila* Psc-containing PcG complex demonstrated the existence of a core set of factors (Pc-ph-Psc-dRING) that are collectively called the PRC1 (Shao et al. 1999). One hypothesis would predict that other factors could assemble with this "basal complex". These factors could regulate the core complex or even target it to particular sites on the DNA. In fact, further purification from *Drosophila* embryos revealed that the PRC1 is associated with Zeste and the dTAFIIs (Saurin et al. 2001). The presence of both sequence specific and basal transcription

factors could be responsible for the targeting of PRC1 to the DNA. The existence of a similar "basal PcG complex" in mammalian cells is hinted at in this report (Saurin et al. 2001). If this complex exists, the role of E2F6 could be to target some proportion of the core Bmi1-containing complex to the promoters of particular target genes (see below).

In this study, we identified Bmi1, Ring1a, and RYBP as E2F6 interacting proteins. Since RYBP was isolated in a yeast-two-hybrid assay and *S. cerevisae* does not possess PcG proteins, it is reasonable to believe that the E2F6/RYBP interaction is direct. However, it is possible that the interactions between E2F6 and the other PcG proteins are bridged by additional factors. To begin to address this problem, we mapped the regions of these proteins responsible for these interactions (Appendix A). Our data are consistent with the existence of a trimolecular E2F6/RYBP/Ring1a complex. The interaction domain on E2F6 that mediates binding to Bmi1 overlapped with the sequences responsible for binding to RYBP and Ring1a. Since Bmi1 has been shown to interact directly with Ring1a (Satijn and Otte 1999b), it is likely that Ring1a facilitates the association between Bmi1 and E2F6. Therefore, these studies have begun to elucidate the nature of the E2F6-containing PcG complex. It will be of interest to determine the regions of E2F6 that mediate the interactions with YY1 and YAF2 and see if these sequences overlap with any of the other interaction sites.

(ii) Mechanisms for DNA binding of the PcG complex

In *Drosophila* embryos, recent results suggest that PcG proteins may simply utilize general transcription factors (such as dTAFIIs and Zeste) to target promoters (Breiling et al. 2001; Saurin et al. 2001). These results leave some unanswered questions, however. First, it is unclear how the use of basal transcription factors could generate specificity in

the genes targeted for PcG-mediated repression. Second, PcG complexes participate in the stable maintenance of transcriptional repression and the exact mechanism that accounts for this stability is still not well understood. Finally, others factors, such as pleiohomeotic, the fly YY1 homologue, have also been implicated in PcG-mediated silencing (Brown et al. 1998). The roles played by these other, perhaps more specific, DNA binding factors remains to be determined.

The mechanisms through which the mammalian PcG complexes recognize DNA are even less understood than those for their *Drosophila* counterparts. During the course of this study several potential DNA binding proteins, including E2F6 (Chapter 3) and YY1 (Brown et al. 1998), have been identified as proteins associated with members of the polycomb group. One model to explain how polycomb complexes recognize the DNA proposes that different compositions of PcG complexes may use distinct DNA binding factors to target particular genes for silencing. Additionally, it has recently been demonstrated that chromodomains, such as those from Pc homologues, can bind to methylated lysine residues in histone proteins (Bannister et al. 2001; Lachner et al. 2001). These data provide another potential mechanism through which PcG complexes can bind to DNA. However, since this mechanism relies on histone modifications, it is conceivable that this methylation could be utilized not to initially target the PcG complex, but instead as a way to mark and/or maintain silenced genes. Some PcG proteins, such as Bmi1, have been shown to dissociate with the DNA during mitosis (Voncken et al. 1999). Perhaps, the PcG complexes could use inheritable marks like methylation to locate silenced genes after mitosis.

E2F6 contains a highly conserved DNA binding domain and exhibits some DNA binding activity, at least *in vitro* (Chapter 2). This property leads us to speculate that E2F6 may provide some DNA recognition function to a subset of PcG complexes. However, we are lacking a key piece of evidence in support of this model. We and others have shown that the DNA binding properties of E2F family members is dependent upon association with DP. Using our panel of monoclonal antibodies, we have been unable to detect the DP proteins in association with the E2F6-containing PcG complex (data not shown). This could reflect either antibody constraints or the absence of the DP proteins in this complex. If E2F6/DP heterodimers are part of the PcG complex, then the promoters of a subset of PcG target genes would be predicted to contain consensus E2F binding sites. If instead, DP proteins are excluded from PcG complexes, E2F6 could be playing a role in DNA targeting to unknown sequences or E2F6 may play no role in sequence specificity at all. Resolving this issue will be a critical feature in understanding the role and mechanism of action of the E2F6-containing PcG complex.

(iii) Implications for the identification of PcG target genes

Another major question in the polycomb field is the identity of the target genes that are silenced by the different PcG complexes. These target genes are best understood in *Drosophila*. Analyzing the phenotypes of PcG mutant *Drosophila* showed that the polycomb complex normally acts to maintain the homeotic (Hox) genes in a silenced state (reviewed in Kennison 1995). Another approach in the search for PcG target genes has been to scan for the presence of polycomb response elements (PREs) in gene promoters. Using this method, PREs were discovered in a number of *Drosophila* segmentation genes, including *engrailed* and *invected* (Orlando et al. 1997; Strutt and

Paro 1997). These studies suggest that, at least in *Drosophila*, PcG complexes regulate a number of genes important for development and differentiation.

The identity of PcG target genes is not as clear in mammalian systems. The analysis of several PcG mutant mouse strains suggests that the mammalian PcG complexes also regulate the activity of the homeotic genes (reviewed in Gould 1997). The isolation of MEFs and hematopoietic cells from several of the PcG deficient mice revealed that these proteins also play a key role in cellular proliferation (van der Lugt et al. 1994; Core et al. 1997; Tetsu et al. 1998; Jacobs et al. 1999). Results presented in this study suggest that the E2F6-containing PcG complex may also participate in the regulation of genes critical for the cell cycle (Chapter 4). E2F6, RYBP, Bmi1 and Ring1a all display a cell cycle regulated expression pattern with the peak of expression coinciding with the G1/S transition. It is still unclear, however, exactly which genes this complex may target. Another unresolved question is whether the cell cycle targets of this PcG complex would need to be silenced every cell cycle. It is conceivable that the PcG proteins are induced during G1/S to be ready to silence the appropriate genes in the daughter cells once S phase and mitosis have occurred.

Studies of the oncogene *Bmi1* have yielded additional candidates for PcG target genes. *Bmi1* was originally identified as an insertion site for Moloney murine leukemia virus that could cooperate with the *Eμ–myc* transgene in the generation of tumors (Haupt et al. 1991; van Lohuizen et al. 1991). The loss of Bmi1 was subsequently shown to result in the derepression of the $p16^{INK4a}$ and $p19^{ARF}$ tumor suppressor genes (Jacobs et al. 1999). Although it is thought that the Bmi1 effects on the *INK4* locus are the result of direct transcriptional repression, this has not been demonstrated. Intriguingly, the $p19^{ARF}$

promoter contains an E2F consensus site (Bates et al. 1998). In this study, we demonstrate that exogenous E2F6 can lead to a downregulation in $p19^{ARF}$ expression (Chapter 4). This supports the idea that E2F6 could be acting to recruit the Bmi1-containing PcG complex to this promoter or at least be contributing to DNA binding specificity. As described above, the validity of this model depends upon whether or not DP is present in the complex.

Formaldehyde cross-linking coupled with chromatin immunoprecipitation (X-CHIP) has been used to map the binding of the PcG proteins to the bithorax cluster of genes (Strutt et al. 1997; Strutt and Paro 1997). This approach could resolve the issue of whether or not E2F6 and/or Bmi1 is present at the $p19^{ARF}$ promoter. In this study, we have generated panels of monoclonal antibodies to E2F6, RYBP and Bmi1 (Chapters 3 and 4). Using these reagents in X-CHIP assays, we should be able to acquire critical information to address whether the Bmi1/E2F6-mediated regulation of the $p19^{ARF}$ promoter is direct or indirect. Additionally, one could use X-CHIP to examine E2F sites in other genes for the presence of E2F6. If E2F6 were found at the promoters of other E2F targets, that finding would suggest E2F6 is capable of binding E2F sites *in vivo* and regulating E2F target genes.

C. The PcG and chromosome condensation

While the exact mechanism by which the mammalian PcG complexes associate with DNA sequences is not well understood, it is widely believed that PcG mediated silencing involves the modification of chromatin structure (reviewed in Mahmoudi and Verrijzer 2001). Purified PRC1 from *Drosophila* embryo extracts can prevent nucleosome

remodeling by SWI/SNF complexes (Shao et al. 1999). These results suggest a hypothesis wherein the PRC1 could maintain silencing by locking the nucleosomes in a conformation that is inaccessible to activating factors.

Another potential link between PcG complexes and chromatin modification has recently been uncovered. The chromodomain motif in HP1 and Pc proteins was shown to bind to histones that had been methylated by SUV39H1 (Bannister et al. 2001; Lachner et al. 2001). In addition to providing insight into potential DNA binding mechanisms of the PcG complex (see above), the presence of a chromodomain also furnishes a possible link between polycomb complexes and heterochromatin. Binding of HP1 to methylated histone tails is hypothesized to initiate the formation of heterochromatin. HP1 proteins can then recruit additional SUV39H1 enzymes causing more histone methylation and a proprogation of HP1-mediated silencing along the chromosome (Bannister et al. 2001). Since the chromodomain of Pc recognizes methylated histones in a similar manner to HP1, its associated complex may be capable of influencing the local chromatin environment.

In this study, we demonstrate that exogenous RYBP can induce the formation of "DNA blob" structures that are reminiscent of condensed chromatin. At this juncture, we have been unable to definitively show that these "blobs" are in fact heterochromatin, since staining of the chromosomes by FISH has not yet been successful. Nevertheless, two models can be envisioned through which overexpression of RYBP would induce the formation of the "DNA blobs". RYBP could be sequestering an inhibitor of chromosome condensation such as RCC1 (Uchida et al. 1990; Seki et al. 1992). Alternatively, RYBP could be nucleating the formation of a complex on DNA that would then drive the

compaction of the DNA. Given the ability of HP1 proteins to induce a similar phenomenon, coupled with the fact that HP1 proteins are found associated with heterochromatin, we investigated whether overexpression of RYBP led to HP1 localizing to the chromosomes. However, antibodies specific for each of the three mammalian HP1 isoforms (Chemicon) did not stain our "DNA blobs" (our unpublished results). Instead, we observed a lack of HP1 staining altogether in those cells. These cells also fail to stain for endogenous Bmi1 and a centromeric marker (CENP-B) (data not shown). This lack of staining suggests that the "DNA blobs" are so compacted that antibodies are unable to penetrate and therefore, no staining is observed.

An alternative approach involves mapping the domains on RYBP that correlate with the induction of the "blobs". We have attempted to do exactly that using our panel of RYBP deletion mutants (Chapter 3). Unfortunately, all of the deletion mutants tested seemed to be mislocalized (diffuse staining throughout the cell) and all failed to induce the "DNA blobs". One clue as to the region of RYBP responsible for the phenomenon comes from our observation that the highly related protein YAF2 fails to induce the "blobs". Therefore, one could use a sequence comparison to engineer a YAF2 protein that induces the "blobs" and an RYBP that does not. Once the region responsible has been identified, the interacting proteins that bind there can be sought.

D. Generation of E2F6 deficient mice

The central questions regarding E2F6 involve uncovering its biological role. Perhaps the most straightforward way to accomplish this goal is to generate an *E2f6* deficient mouse. If the *in vivo* role of E2F6 is linked to the Bmi1-containing polycomb complex,

the *E2f6*$^{-/-}$ mice should display skeletal abnormalities reminiscent of the other "late-acting" PcG knockout mice. One possible complication that could arise is if *E2f6*$^{-/-}$ embryos are inviable. YY1 deficient mice fail to survive beyond E6.5 (Donohoe et al. 1999) and we have shown that E2F6 can associate with YY1 (Appendix B). Additionally, *E2f6* transcripts can be detected as early as the four-cell stage (Palena et al. 2000). These observations point to the possibility of a severe early embryonic phenotype. However, even if these mice die this early, we will have discovered not only that *E2f6* is an essential gene, but that it also most likely plays an additional role from any that involve the Bmi1-containing PcG complex.

If the phenotype of E2F6 is consistent with a role in the Bmi1-containing PcG complex, then the isolation of *E2f6*$^{-/-}$ cells should be possible. By examining the senescence phenotype of these cells, one could test whether E2F6 plays an analogous role to Bmi1 in the control of cellular proliferation. Furthermore, p19^{ARF} expression could be examined in *E2f6* deficient MEFs. Besides investigating the small list of proposed PcG target genes, these *E2f6*$^{-/-}$ cells could be used in an unbiased microarray screen to identify many possible target genes.

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Appendix A

Mapping the interactions between E2F6 and its associated PcG proteins

Jeffrey M. Trimarchi and Jacqueline Lees

E2F6 is the most recently identified member of the E2F family of transcription factors. While E2F6 possesses considerable homology to the other E2Fs in its DNA binding and DP dimerization domains, it lacks the sequences responsible for pocket protein binding and transactivation in the other E2Fs. E2F6, therefore, is the founding member of a third subgroup of the E2F family and will likely display distinct biological properties. In an effort to identify the role of E2F6 *in vivo*, we performed a screen for E2F6 interacting proteins. Using a yeast-two hybrid assay, we found that E2F6 interacts with RYBP, a member of the mammalian polycomb complex (PcG) (see Chapter 3). We subsequently showed that the marked-box domain of E2F6 and the C-terminus of RYBP are the critical regions for this interaction. In the same study, we went on to demonstrate that E2F6 associates with two additional PcG proteins, Ring1a and Bmi1. However, the domains necessary for these interactions were not identified.

In order to more fully define the interactions among E2F6, RYBP, Ring1a, and Bmi1, we used a deletion mutant strategy to map the regions responsible for each pairwise association. To determine the sequences in Ring1a that mediate the interaction with E2F6, we constructed a panel of Ring1a deletion mutants (Fig. A1). As before, the expression of these mutants was confirmed by Western blotting (Fig. A1). Although the

N-terminal RING finger domain of Ring1a has been hypothesized to be involved in protein-protein interactions, none of the previously identified Ring1a-interacting proteins required these sequences for binding (Satijn and Otte 1999). Likewise, the association with E2F6 did not require the RING finger domain. A construct of Ring1a spanning residues 200-377 was sufficient to mediate the interaction with E2F6 (Fig. A1). Further mapping revealed that E2F6 binds robustly to Ring1a constructs encompassing either residues 200-284 or residues 279-377 (Fig. A1). Thus, the C-terminus of Ring1a is capable of mediating an interaction with E2F6.

To map the sequences in E2F6 that are required for Ring1a binding, we utilized the same set of E2F6 deletion mutants as in Chapter 3 (Fig. A2). For each of these experiments, the expression of the E2F6 deletion mutants was confirmed by Western blotting of whole cell extracts (Fig. A2). As was the case with RYBP, the distinct N-terminal 62 amino acids of E2F6 were dispensable for the association with Ring1a (Fig. A2). Deleting an additional 66 amino acids from the N-terminus of E2F6 likewise had no effect on Ring1a binding. Removal of the C-terminal 41 amino acids (residues 240-281), however, abolished the interaction between E2F6 and Ring1a (Fig.A2). Our previous studies have mapped the RYBP binding site to the marked box domain of E2F6 (residues 179-240) (Chapter 3 Fig. 2). Thus, the Ring1a binding site of E2F6 encompasses the RYBP binding domain, but is dependent upon additional C-terminal residues. These data also provide a potential explanation for why Ring1a was not identified in our yeast-two-hybrid assay, as the C-terminus of E2F6 was not included.

Ring1a has previously been shown to bind to RYBP in a yeast-two-hybrid assay, suggesting that the interaction between these two proteins is direct (Garcia et al. 1999).

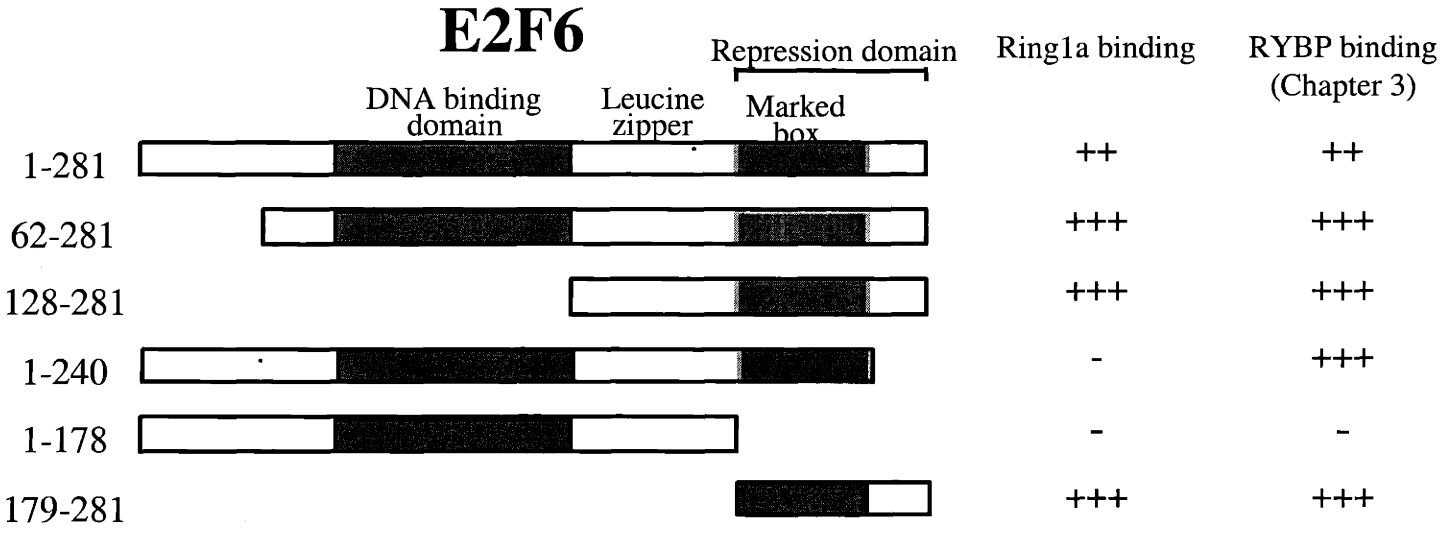
Since our assay relies on expression in mammalian cells, we cannot rule out that the requirement of the RYBP interaction site for E2F6 to bind Ring1a reflects the presence of RYBP in trimolecular complexes. In addition, previous studies have yielded unclear results about the minimal sequences required for an interaction between RYBP and Ring1a (Garcia et al. 1999). We, therefore, used our panel of RYBP mutants (Chapter 3) to map the sequences in RYBP that mediate its interaction with Ring1a. Since zinc finger domains often mediate protein-protein interactions, we began our analysis by deleting the first 72 amino acids of RYBP. This truncated RYBP protein bound to Ring1a with the same affinity as the full-length protein, indicating that this domain is dispensable for the interaction (Fig. A3). Using an N-terminal (73-143) and a C-terminal (144-228) mutant of RYBP, we were able to further narrow the region of Ring1a binding to residues 144-228 (Fig. A3). Since constructs containing additional deletions of RYBP sequence did not express detectable protein products, we used a panel of C-terminal deletions in the context of full-length RYBP to more fully define the region responsible for interaction with Ring1a. Deletion of the 21 amino acids from the C-terminus of RYBP (1-207) did not affect its ability to associate with Ring1a (Fig, A3). However, deletion of 42 more amino acids (1-165) completely abolished the association. Thus, in our experiments, association with Ring1a requires sequences contained within amino acids 165-207 of RYBP.

Previously, Ring1a has been shown to interact with many PcG proteins including RYBP, Bmi1 and hPc2 (Garcia et al. 1999; Satijn and Otte 1999). Among these proteins there are two, RYBP and hPc2, that interact through the C-terminus of Ring1a (Garcia et al. 1999; Satijn and Otte 1999). The interaction with RYBP requires residues 201-377

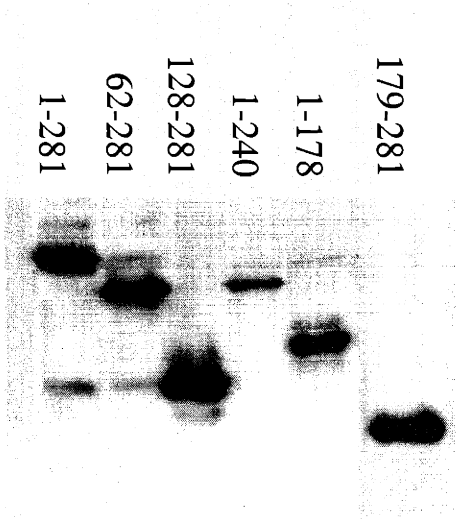
while residues 230-377 mediate the interaction with hPc2. In this study, we show that the interaction between E2F6 and Ring1a requires sequences (amino acids 200-284) embedded within the binding domains of these other proteins. Similarly, the Ring1a interaction site on RYBP maps to sequences adjacent to the E2F6 binding region and the Ring1a association domain on E2F6 maps to sequences near the RYBP interacting site. Taken together these results provide evidence for a model in which E2F6/RYBP/Ring1a associate in a multiprotein PcG complex, where each protein contributes some sequences critical for the assembly of the complex.

Finally, we mapped the sequences required for association between E2F6 and Bmi1. For this experiment, we were only able to test two deletion mutants of E2F6 with Bmi1 (Fig. A4). However, even the smallest E2F6 construct (179-281) assayed was able to interact with Bmi1 (Fig. A4). This construct is comprised of the RYBP interaction domain (marked box) and the Ring1a interaction domain (C-terminus). These data suggest that it is unlikely that RYBP, Ring1a and Bmi1 would all be capable of directly interacting with E2F6 in the same complex. Using a yeast-two-hybrid assay, Ring1a and Bmi1 have been shown previously to interact directly (Satijn and Otte 1999). Given this result and those presented in this study, it is conceivable that E2F6, RYBP, Ring1a and Bmi1 could still participate in the same complex if some of the protein-protein interactions were indirect.

Figure A1

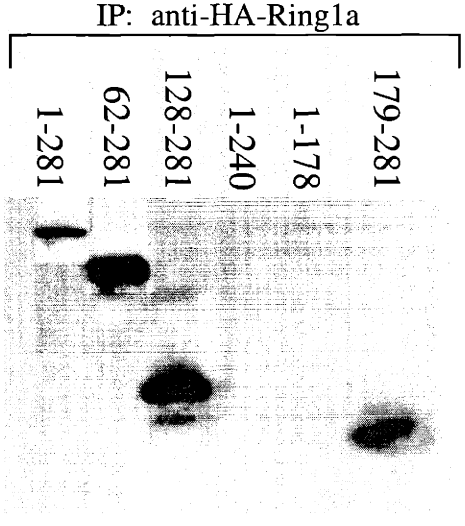


Expression of E2F6 mutants



WB:anti-E2F6

Ring1a binding



WB:anti-E2F6

Figure A2

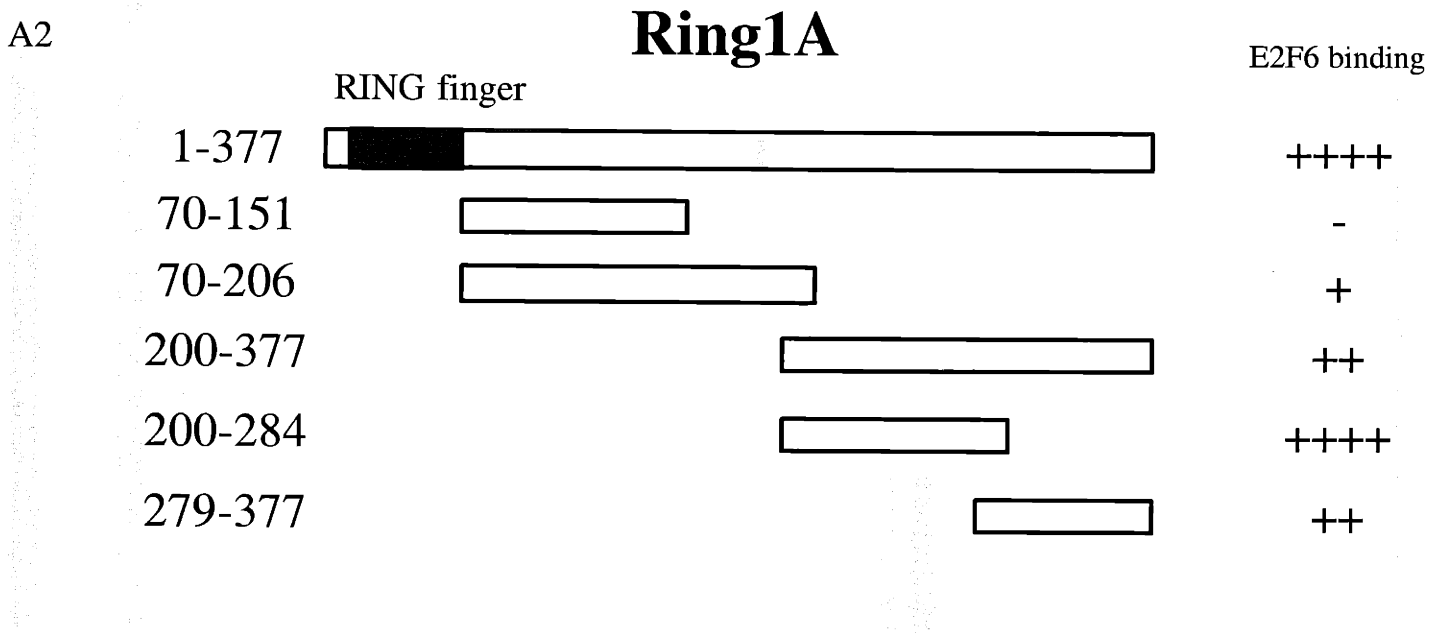
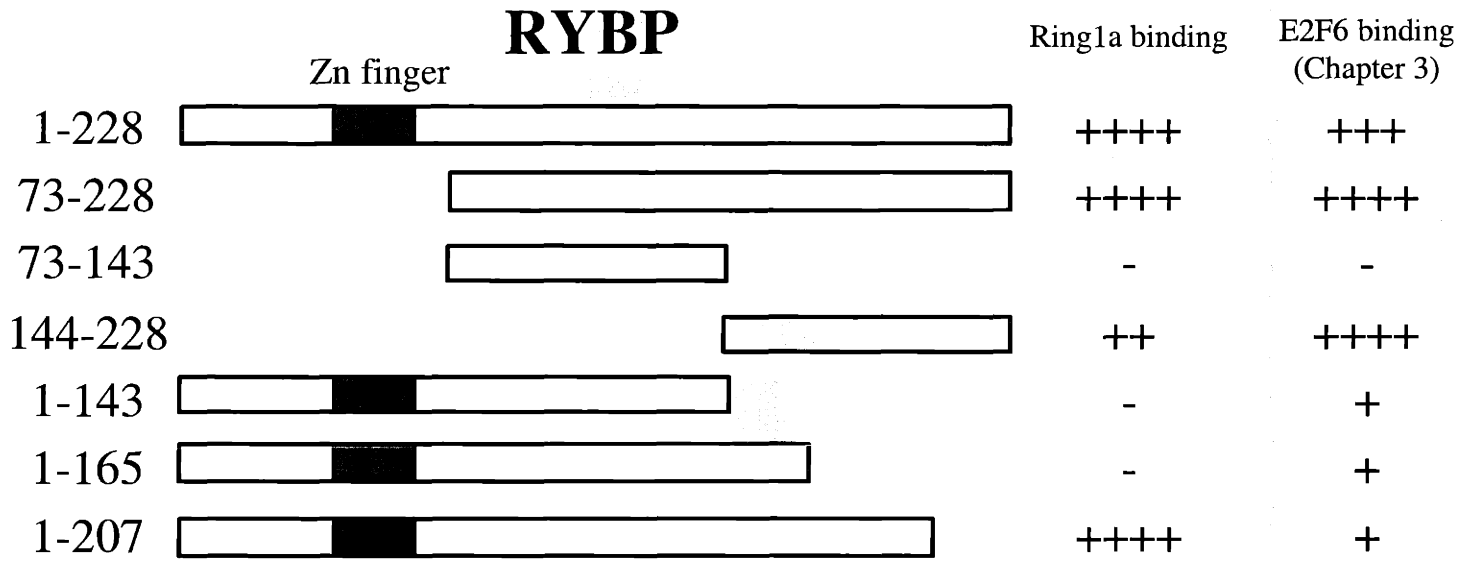
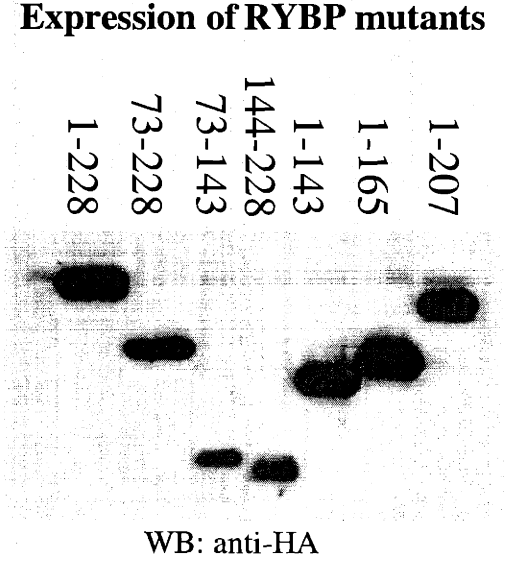

Ring1A
RING finger
E2F6 binding
1-377
++++
70-151
-
70-206
+
200-377
++
200-284
++++
279-377
++
Expression of Ring1a mutants
1-377
70-151
70-206
200-377
200-284
279-377
WB: anti-HA
E2F6 Binding
IP : anti-HA-Ring1a
WCE
-
-
1-377
70-151
70-206
200-377
200-284
279-377
WB: anti-E2F6

Figure A3


RYBP
Zn finger
Ring1a binding
E2F6 binding
(Chapter 3)
1-228
++++
+++
73-228
++++
++++
73-143
-
-
144-228
++
++++
1-143
-
+
1-165
-
+
1-207
++++
+


Expression of RYBP mutants
1-228
73-228
73-143
144-228
1-143
1-165
1-207
WB: anti-HA

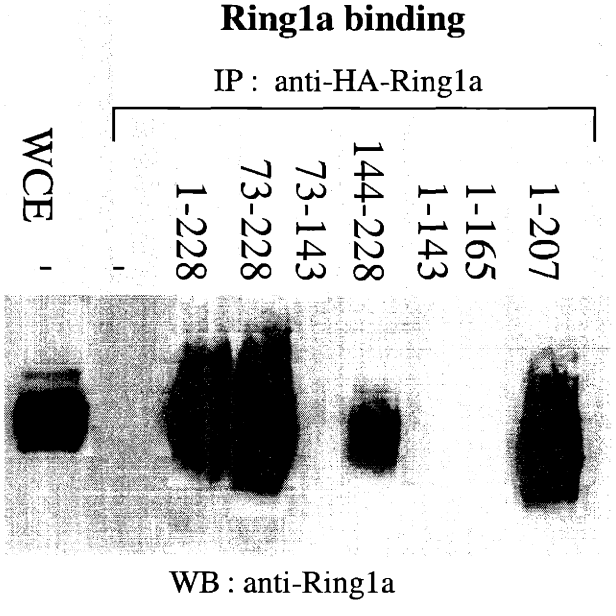
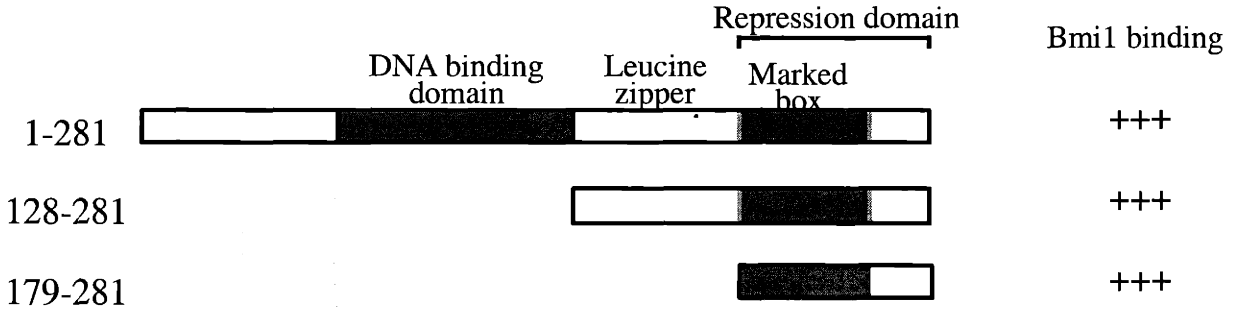
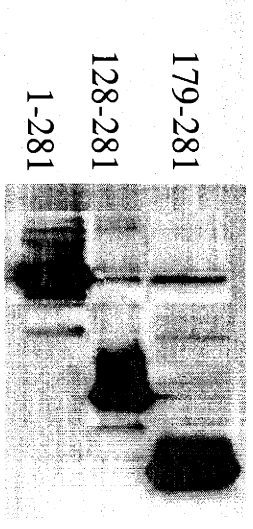

Ring1a binding
IP : anti-HA-Ring1a
WCE
-
-
1-228
73-228
73-143
144-228
1-143
1-165
1-207
WB : anti-Ring1a

Figure A4

E2F6



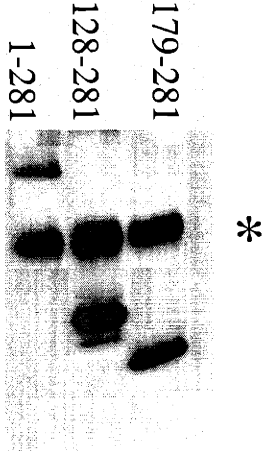
Expression of E2F6 mutants



WB: LLF6-2

Bmi1 binding

IP: anti-HA-Bmi1



WB : LLF6-2

Figure Legends

Fig. A1 Mapping the E2F6-Ring1a interaction domain. C33-A cells were transiently transfected with expression vectors encoding full length HA-Ring1a and the panel of E2F6 mutants shown. Expression of the transfected proteins was confirmed by Western blotting a fraction of the cell lysate. The remainder was immunoprecipitated with an anti-HA antibody (12CA5) and then immunoblotted with a pool of anti-E2F6 monoclonal antibodies (LLF6-1, LLF6-2, and LLF6-3) that recognizes all of the E2F6 mutants. An interaction summary is depicted at the top.

Fig. A2 Mapping the Ring1a-E2F6 interaction domain. C33-A cells were transiently transfected with expression vectors encoding full length E2F6 and the panel of HA-Ring1a mutants shown. Expression of the transfected proteins was confirmed by Western blotting a fraction of the cell lysate. The remainder was immunoprecipitated with an anti-HA antibody (12CA5) and then immunoblotted with an anti-E2F6 monoclonal antibody (LLF6-2). An interaction summary is depicted at the top.

Fig A3 Mapping the RYBP-Ring1a interaction domain. C33-A cells were transiently transfected with expression vectors encoding full length Ring1a and the panel of HA-RYBP mutants shown. Expression of the transfected proteins was confirmed by Western blotting a fraction of the cell lysate. The remainder was immunoprecipitated with an anti-HA antibody (12CA5) and then immunoblotted with an anti-Ring1a polyclonal antibody (ASA8). An interaction summary is depicted at the top.

Fig A4 Mapping the E2F6-Bmi1 interaction domain. C33-A cells were transiently transfected with expression vectors encoding full length HA-Bmi1 and the E2F6 mutants shown. Expression of the transfected proteins was confirmed by Western blotting a fraction of the cell lysate. The remainder was immunoprecipitated with an anti-HA antibody (12CA5) and then immunoblotted with a pool of anti-E2F6 monoclonal antibodies (LLF6-1, LLF6-2, LLF6-3) that recognizes the E2F6 mutants shown. An interaction summary is depicted at the top. The Ig light chain is denoted by an asterisk.

References

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Appendix B

E2F6 and the transcription factor YY1 associate with common partners

Jeffrey M. Trimarchi and Jacqueline Lees

E2F6 is the most recently identified member of the E2F family of transcription factors. While E2F6 possesses considerable homology to the other E2Fs in its DNA binding and DP dimerization domains, it lacks the sequences that mediate pocket protein binding and transactivation in the other E2Fs. E2F6, therefore, is the founding member of a third subgroup of the E2F family and will likely display distinct biological properties. In an effort to identify the role of E2F6 *in vivo*, we performed a screen for E2F6 interacting proteins. Using a yeast-two hybrid assay, we found that E2F6 interacts with RYBP, a member of the mammalian polycomb complex (PcG) (see Chapter 3). Furthermore, we showed that E2F6 could associate with several additional members of the Bmi1 containing polycomb complex. Since the exact mechanism through which PcG proteins interact with their DNA response elements is currently unknown, the identification of E2F6 as a member of the polycomb complex suggested that it could contribute to a targeting function.

Recently, the mammalian transcription factor YY1, and its *Drosophila* counterpart, *pleiohomeotic*, also have been found to be involved in the polycomb complex (Brown et al. 1998; Satijn et al. 2001). Additionally, YAF2, a protein highly related to RYBP, has been isolated in a yeast-two-hybrid screen with YY1 (Kalenik et al. 1997). While YY1 is

a DNA binding protein implicated in the polycomb complex, it is clear that YY1 cannot account for all the DNA binding activity of the mammalian PcG complex (Brown et al. 1998). Additionally, a recent report showed that YY1 can only associate with members of the Eed/Ezh complex and not with proteins from the Bmi1 containing complex (Satijn et al. 2001).

We wished to ascertain whether YY1 and E2F6 could participate in the same PcG complex. To address this question, C33-A cells were transiently transfected with expression vectors encoding E2F6, RYBP, Ring1A, or Bmi1 in the presence or absence of a HA-tagged version of YY1. The cells were then labeled with [^{35}S]methionine and immunoprecipitated with antibodies specific for E2F6 (LLF6-1), RYBP (LLRYBP-1), Ring1A (ASA8), Bmi1 (LLBmi1-1) or the HA tag (12CA5). YY1 was not found to associate with E2F6 (Fig. B1A), RYBP (Fig. B1B), or Bmi1 (Fig. B1D) in this assay. This result is consistent with the recent reports that found YY1 could interact with the components of the embryonic ectoderm development (eed)-containing polycomb complex, but not with the proteins from the Bmi1-containing complex (Satijn et al. 2001). However, contrary to this report, we find that Ring1A and YY1 interact robustly (Fig. B1C). Also, the finding that YY1 and RYBP fail to interact is contradictory to a previous report that found RYBP and YY1 could interact at least *in vitro* (Garcia et al. 1999). These results suggest that YY1 is capable of associating with Ring1a, a component of the E2F6- and Bmi1-containing polycomb complexes. In addition, these data suggest that we have to re-examine which proteins can actually associate with YY1.

We also tested for an interaction between the endogenous YY1 and E2F6 proteins. Whole-cell lysates from ML-1 cells were incubated with an anti-YY1 antibody (Santa

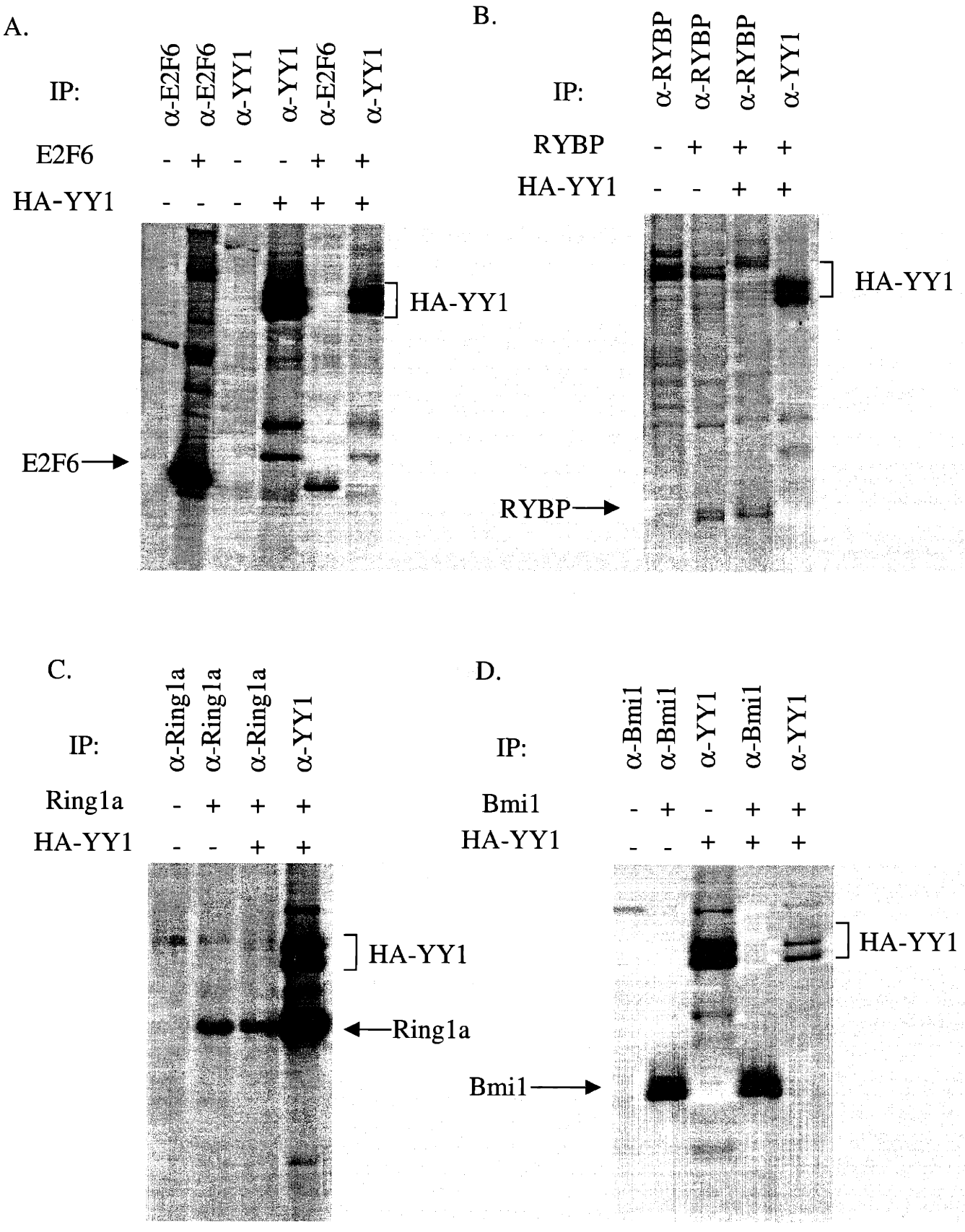
Cruz, sc-281), and the resulting immunocomplexes were resolved by SDS/PAGE and then immunoblotted with an anti-E2F6 monoclonal antibody (LLF6-2). No E2F6 protein was recovered by the preimmune serum, but we detected a low and reproducible amount of E2F6 present in the YY1 immunoprecipitates (Fig. B2). Immunoprecipitations with antibodies directed against several PcG proteins are included for comparison (Fig. B2). Due to reagent constraints, we were unable to determine whether endogenous Ring1a and YY1 can associate. Taken together, these data indicate that E2F6 and YY1 can associate in the same complex, perhaps bridged by Ring1a.

We previously identified RYBP as an E2F6 interacting protein (see Chapter 3). Since RYBP and YAF2 are highly related proteins, we wondered whether E2F6 could interact with YAF2 as well. C33-A cells were transiently transfected with expression vectors encoding E2F6 and an HA-tagged version of YAF2. The cells were labeled with [^{35}S]methionine and the resultant complexes recovered with monoclonal antibodies specific for either E2F6 (LLF6-1) or the HA tag (12CA5). Similar to our results with E2F6 and RYBP (see Chapter 3), we consistently observed YAF2 in the anti-E2F6 immunoprecipitate, although at low levels (Fig. B3 lane 4). Additionally, the anti-HA tag antibody was able to recover both HA-YAF2 and E2F6 (Fig. B3 lane 5). Thus, E2F6 and YAF2 can form a complex that is recovered in the same fashion as the E2F6/RYBP complex.

The mammalian transcription factor YY1 can interact with the Eed/Ezh containing polycomb complex (Satijn et al. 2001). This result has important implications for the mechanism by which this complex could bind to DNA. In fact, consensus binding sites for the *Drosophila* YY1 homologue (Pho) have been found in a wide variety of polycomb

response elements (PREs) (Brown et al. 1998; Mihaly et al. 1998). E2F6 represents another potential DNA binding protein that associates with the PcG complex. In experiments detailed in this appendix, we demonstrate that E2F6 and YY1 share at least two common interacting partners.

Figure B1


A.
IP:
α-E2F6
α-E2F6
α-YY1
α-YY1
α-E2F6
α-YY1
E2F6 - + - - + +
HA-YY1 - - - + + +
HA-YY1
E2F6
B.
IP:
α-RYBP
α-RYBP
α-RYBP
α-YY1
RYBP - + + +
HA-YY1 - - + +
HA-YY1
RYBP
C.
IP:
α-Ring1a
α-Ring1a
α-Ring1a
α-YY1
Ring1a - + + +
HA-YY1 - - + +
HA-YY1
Ring1a
D.
IP:
α-Bmi1
α-Bmi1
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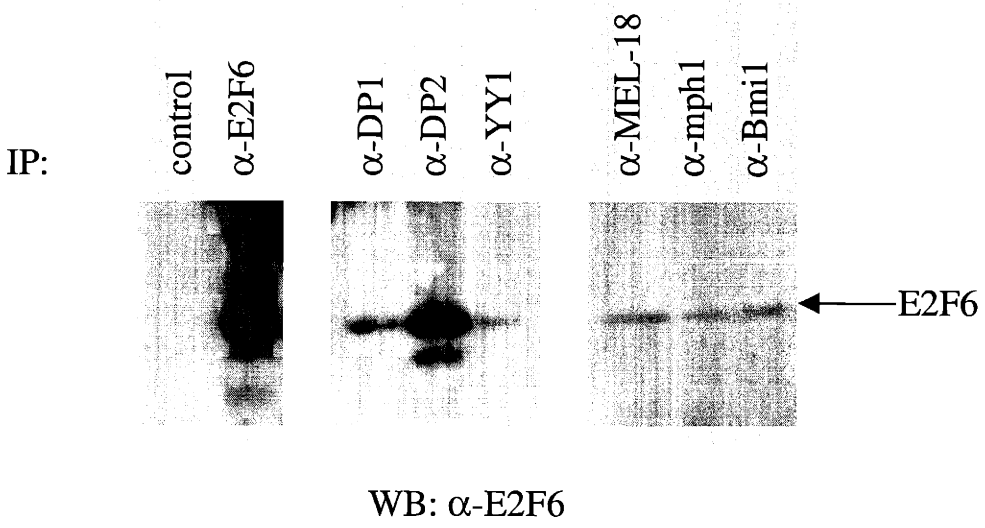
HA-YY1 - - + + +
HA-YY1
Bmi1

Figure B2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α-Bmi1
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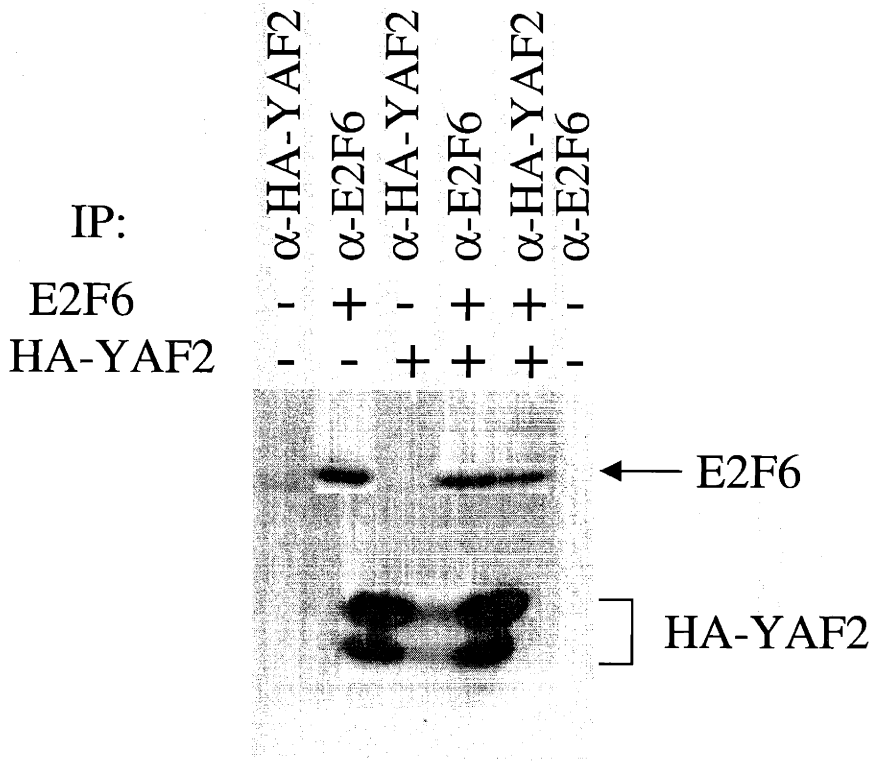
WB: α-E2F6

Figure B3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E2F6
- + - + + -
HA-YAF2
- - + + + -
E2F6
HA-YAF2

Figure Legends

Fig.B1. Ring1A can associate with YY1. (A) C-33A cells were transiently transfected with expression vectors encoding E2F6 and/or HA-tagged YY1, labeled with [^{35}S]methionine, and immunoprecipitated with the indicated antibodies. (B) C-33A cells were transfected as in (A) with vectors encoding either RYBP or HA-tagged YY1 and immunoprecipitated with the indicated antibodies. (C) C-33A cells were transfected as in (A) with Ring1A or HA-YY1 and immunoprecipitated with the indicated antibodies. (D) C-33A cells were transfected as in (A) with Bmi1 or HA-YY1 and immunoprecipitated with the indicated antibodies.

Fig. B2. E2F6 can associate with YY1. ML-1 cell lysates were immunoprecipitated with control (12CA5), anti-E2F6 (LLF6-1), anti-DP1 (Santa Cruz, sc-), anti-DP2 (Santa Cruz, sc-), anti-YY1 (Santa Cruz, sc-), anti-MEL-18 (M. van Lohuizen), anti-mph1 (M. van Lohuizen) or anti-Bmi1 (M. van Lohuizen), resolved by SDS/PAGE, and immunoblotted with an additional anti-E2F6 monoclonal antibody (LLF6-2).

Fig. B3. YAF2 is an E2F6 interacting protein. C-33A cells were transiently transfected with expression vectors encoding E2F6 and/or HA-YAF2, labeled with [^{35}S]methionine, and immunoprecipitated with monoclonal antibodies specific for E2F6 (LLF6-1) or the HA tag (12CA5).

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Appendix C

_E2f3_ is critical for normal cellular proliferation.

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Author's contribution: Figures 1B, 2B, 6, 7

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Abstract

E2F is a family of transcription factors that regulate both cellular proliferation and differentiation. To establish the role of E2F3 *in vivo,* we generated an *E2f3* mutant mouse strain. E2F3-deficient mice arise at one quarter of the expected frequency, demonstrating that E2F3 is important for normal development. To determine the molecular consequences of E2F3 deficiency, we analyzed the properties of embryonic fibroblasts derived from *E2f3* mutant mice. Mutation of *E2f3* dramatically impairs the mitogen induced, transcriptional activation of numerous E2F-responsive genes. We have been able to identify a number of genes, including *B-myb, cyclin A, cdc2, cdc6* and *DHFR*, whose expression is dependent upon the presence of E2F3 but not E2F1. We further show that a critical threshold level of one or more of the E2F3-regulated genes determines the timing of the G_1/S transition, the rate of DNA synthesis and thereby the rate of cellular proliferation. Finally, we show that E2F3 is not required for cellular immortalization but is rate limiting for the proliferation of the resulting tumor cell lines. We conclude that E2F3 is critical for the transcriptional activation of genes that control the rate of proliferation of both primary and tumor cells.

INTRODUCTION

The E2F transcription factors control the cell cycle dependent expression of genes that are essential for cellular proliferation (reviewed in Dyson 1998; Helin 1998). E2F activity is regulated by the retinoblastoma protein (pRB), a tumor suppressor that is functionally inactivated in most, if not all, human tumors (reviewed in Weinberg 1992; Dyson 1998). pRB binds to E2F during the G_1 phase of the cell cycle. This association inhibits the transcriptional activity of E2F and the resulting complex actively represses E2F-responsive genes by recruiting histone deacetylases to the promoter (reviewed in Dyson 1998; Brehm and Kouzarides 1999). At the G_1/S transition, pRB is phosphorylated by the cyclin dependent kinases and the released E2F now activates transcription. In this manner, E2F participates in both the repression and activation of E2F responsive genes.

pRB belongs to a family of proteins, called the pocket proteins, that includes two additional members, p107 and p130 (reviewed in Dyson 1998). Like pRB, p107 and p130 can bind to E2F complexes, inhibit E2F-mediated transactivation and enforce the active transcriptional repression of E2F-responsive genes (Starostik et al. 1996; Zwicker et al. 1996; Iavarone and Massague 1999). However, the biological properties of p107 and p130 clearly differ from those of pRB (reviewed in Mulligan and Jacks 1998). Mutations within *p107* or *p130* are rarely detected in human tumors and they do not increase the tumor predisposition of mutant mouse strains. Moreover, the homozygous mutation of *Rb* causes developmental defects that are distinct from those resulting from the combined loss of *p107* and *p130*. It is widely believed that the differential

developmental and tumor suppressive roles of pRB, p107 and p130 arise from differences in the way in which they regulate E2F.

To date, eight genes encoding components of E2F have been cloned (reviewed in Dyson 1998). Their protein products can be subdivided into two groups, the E2Fs (1 through 6) and the DPs (1 and 2). Over-expression studies indicate that E2F and DP must heterodimerize to generate functional E2F activity. Although the DP subunit is critical for activity, the functional specificity of the E2F•DP complex is determined by the E2F subunit (reviewed in Dyson 1998). The E2F family can be divided into three distinct subgroups, based on both sequence homology and functional properties.

The first subclass contains E2F1, E2F2 and E2F3. When complexed with DP, these E2Fs each have high transcriptional activity and are sufficient to induce quiescent cells to enter S phase (DeGregori et al. 1997; Lukas et al. 1997; Verona et al. 1997). DP•E2F1, DP•E2F2 and DP•E2F3 complexes are specifically regulated by pRB, and not p107 or p130, and the timing of their release from pRB correlates with the timing of activation of E2F-responsive genes (Lees et al. 1993; Moberg et al. 1996). E2F4 and E2F5 represent the second E2F subclass. The DP•E2F4 and DP•E2F5 species are very poor transcriptional activators and they are unable to induce quiescent cells to enter S-phase (Lukas et al. 1996; Muller et al. 1997; Verona et al. 1997). Instead, E2F4 and E2F5 are thought to be important in the repression of E2F-responsive genes through their ability to recruit pRB, p107 and p130 and the associated histone deacetylases (reviewed in Dyson 1998; Helin 1998). E2F6 represents the final E2F subclass (Cartwright et al. 1998; Gaubatz et al. 1998; Trimarchi et al. 1998). E2F6 lacks the sequences required for transcriptional activation or pRB family binding and it can inhibit the transcription of

E2F-responsive genes. From this point on, we will use E2F1, 2, 3 etc to refer to individual E2F proteins, "free" E2F to refer to the E2F•DP complexes and E2F activity to refer to the total pool of the endogenous "free" and pocket protein-containing E2F•DP complexes.

The individual E2F proteins are thought to have different target gene specificities that will account for the different biological properties of pRB, p107 and p130. Potential specificity has been investigated using three different approaches (reviewed in Helin 1998). First, a combination of classic promoter mapping and *in vivo* footprinting have been used to compare the relative contribution of repression (by pocket protein•E2F complexes) and activation (by free E2F complexes) in regulating the activity of individual promoters. These studies concluded that many E2F-responsive genes, including *B-myb*, *cdc2*, *cyclin* E, *cyclin A* and *E2F-1*, are regulated primarily by repressive E2F complexes (Dalton 1992; Lam and Watson 1993; Neuman et al. 1994; Tommasi and Pfeifer 1995; Huet et al. 1996; Zwicker et al. 1996; Le Cam et al. 1999). In contrast, the cell cycle regulation of other E2F-responsive genes (e.g. *DHFR*) seems to be largely dependent upon the presence of activating E2F species (Means et al. 1992; Wade et al. 1992).

In the second approach, a variety of over-expression systems have been used to compare the ability of individual E2F family members to activate the transcription of either endogenous or co-expressed E2F responsive genes (DeGregori et al. 1997; Vigo et al. 1999). These studies have revealed significant differences in the specificity of target gene activation. However, the identity of the E2F-specific targets varies considerably from one study to the next, suggesting that it is highly system-dependent.

The third approach has utilized mutant mouse embryonic fibroblasts (MEFs) to determine how loss of pRB, p107 and/or p130 affects the regulation of known E2F-responsive genes (Herrera et al. 1996; Hurford et al. 1997). These studies demonstrated that p107 and p130 have overlapping functions and together regulate a subset of E2F-responsive genes that are distinct from the pRB-regulated targets. This specificity directly supports the notion that pRB, p107, and p130 regulate E2F in distinct ways. This is presumed dependent upon the ability of the pocket proteins to bind to different E2F family members.

To date, mutant mouse models have been generated for two of the E2F family members. *E2f5* $^{-/-}$ mice die from hydrocephalus caused by excessive secretion of cerebral spinal fluid (Lindeman et al. 1998). This phenotype appears due to a defect in differentiation rather than proliferation. *E2f1* $^{-/-}$ mice are viable and fertile but they develop tissue abnormalities, including testicular atrophy, exocrine gland dysplasia and a defect in thymus apoptosis (Field et al. 1996; Yamasaki et al. 1996). In addition, these mice also develop a broad spectrum of late onset tumors, suggesting that E2F1 can act as a tumor suppressor *in vivo* (Yamasaki et al. 1996). Analyses of *Rb* $^{-/-}$;*E2f1* $^{-/-}$ mice suggest that E2F1 accounts for much of the inappropriate p53-dependent apoptosis and approximately one half of the ectopic S phase entry in *Rb* $^{-/-}$ embryos (Tsai et al. 1998). Consistent with these observations, the absence of E2F1 significantly reduces formation of tumors in *Rb* $^{+/-}$ mice (Yamasaki et al. 1998). These mouse models confirm that individual members of the E2F subclasses have very different biological properties. However, it is unclear how these differences relate to the target specificity of the different E2Fs *in vivo*.

In this study, we have used *E2f3* mutant mouse strains and the resulting *E2f3* mutant cell lines to investigate the role of E2F3 in normal cell cycle regulation. We show that E2F3 plays a crucial role in mediating the normal cell cycle dependent activation of most known E2F-responsive genes and the reduced expression of one or more of these genes in E2F3 deficient cells causes specific defects in the initiation and progression of DNA synthesis. As a result, E2F3 acts in a dose-dependent manner to control the rate of proliferation of both primary and immortalized cells.

RESULTS

E2f3 is critical for full neonatal viability

To establish the role of E2F3 in cell cycle control, we used standard gene-targeting techniques to generate *E2f3*-deficient mice. We functionally inactivated the *E2f3* gene in embryonic stem (ES) cells by introducing an in-frame termination codon immediately prior to the nuclear localization signal (NLS), and replacing the genomic sequences encoding the NLS, cyclin A binding, DNA binding and the leucine zipper domains (amino acids 134 -294) with a neomycin resistance marker (Figure 1A). After electroporation and G418 selection, correctly targeted $E2f3^{+/-}$ ES cell lines were used to generate chimeric animals. Two independent cell lines (F3-1-1 and F3-2-13) were used to transmit the mutation into the germline. The following data was obtained from the analysis of mice and cells derived from ES clone F3-1-1, although both lines showed identical phenotypes.

To assess the role of E2F3 in normal development, we intercrossed the $E2f3^{+/-}$ animals. In this mixed (C57BL/6 x 129/sv) strain background, we were able to detect viable $E2f3^{-/-}$ animals at weaning, however, these were not present at the expected frequency (Table 1; $\chi^2 = 47.8$, $p = 0.005$). Instead, viable $E2f3^{-/-}$ animals arose at approximately one quarter of the predicted number. Preliminary backcrosses suggest that the partial penetrance of this phenotype is due to the presence of one or more strain-specific modifiers (our unpublished observations). We are still investigating the

phenotypes of the *E2f3*$^{-/-}$ animals and the nature of the modifier effect but these studies indicate that E2F3 is critical for full viability.

Loss of E2F3 does not affect other E2F species

We have used the *E2f3* mutant animals to investigate the molecular consequences of E2F3-deficiency and the role of E2F3 in cell cycle control. Mouse embryonic fibroblasts (MEFs) were isolated from the progeny of *E2f3*$^{+/-}$ crosses at embryonic day 13.5. Initially, we examined how the mutation of E2F3 affects the endogenous E2F species. Western blot analysis showed that the homozygous mutation of *E2f3* completely abolishes expression of the E2F3 protein, confirming that this mutation is a null (Figure 1B). We then compared the relative levels and composition of the other E2F complexes using gel shift analysis (EMSA) of whole cell extracts. In wild-type cells, the majority of E2F activity (about 70%-80%) was generated by pocket-protein bound rather than "free" E2F species (Figure 1C, lane 1). To facilitate the detection of the individual E2F family members, we treated the whole cell extracts with sodium deoxycholate (DOC) to dissociate the pocket proteins from the E2F•DP complexes. In wild-type MEFs, addition of anti-E2F-4 antibodies shifts over 70% of the released E2F activity. E2F1, E2F2 and E2F5 were present at low to undetectable levels (Figure 1C and data not shown). In contrast, the anti-E2F-3 antibodies recognized a minor species (representing approximately 10% of total E2F activity) in the wild-type MEF extracts (Figure 1C, compare lanes 2 and 6). Supershift analysis of non-DOC treated extracts showed that this was largely derived from the pRb•E2F complex (data not shown).

Consistent with the complete absence of E2F3 protein, we observed no E2F3 species in the *E2f3*$^{-/-}$ MEFs in either the absence (data not shown) or the presence of DOC. Apart from this change, we did not detect any significant alteration in the relative levels of the other E2F complexes. Thus, at least at a qualitative level, the homozygous mutation of *E2f3* completely disrupts the relevant E2F3 complexes without any apparent compensation by the other E2F family members.

E2f3$^{-/-}$ cells have a proliferation defect

We next wished to determine whether the loss of E2F3 affected the rate of cellular proliferation. For these experiments, passage 4 MEFs, derived from wild-type, *E2f3*$^{+/-}$ and *E2f3*$^{-/-}$ littermates, were cultured under either high or low density conditions. At high density, the *E2f3*$^{-/-}$ MEFs grew considerably less well than their wild-type counterparts (Figure 2A). The severity of the proliferation defect varied from one preparation of *E2f3*$^{-/-}$ MEFs to the next but the average doubling time was approximately twice that of wild-type littermate controls. The *E2f3*$^{+/-}$ MEFs also exhibited a range in their growth rates; some grew at rates indistinguishable from wild-type (Figure 2A), whereas others grew slightly slower (data not shown). This phenotypic variation only occured between mutant MEF lines isolated from different embryos and never the same embryo (data not shown), arguing that it results from genetic variation in the individual mixed (C57BL/6 x 129/sv) background embryos. As described below, we have exploited this variation to dissect the molecular basis of the proliferation defect.

The proliferation defect of the *E2f3*$^{-/-}$ cells was more apparent under low density culture conditions (Figure 2B). While some of the *E2f3*$^{-/-}$ MEF cell lines divided at a

greatly reduced rate, a significant proportion did not proliferate at all. There was little or no difference in the level of apoptosis observed in wild-type and *E2f3*$^{-/-}$ MEFs and there was also no evidence to suggest that the *E2f3*$^{-/-}$ cells reach the end of their proliferative capacity sooner than the wild-type controls (data not shown). This suggests that the proliferation defect of the *E2f3* mutant cells is due to a defect in cell division rather than the induction of apoptosis or premature senescence. As in the high density experiments, some of the *E2f3*$^{+/-}$ MEFs grew as well as wild-type cells, while others have a phenotype that is intermediate between that of the wild-type and *E2f3*$^{-/-}$ cells. Thus E2F3 plays a key role in controlling the rate of proliferation of MEFs in a dose-dependent manner.

E2f3$^{-/-}$ cells have a cell cycle defect

To understand the nature of the proliferation defect, we compared the cell cycle progression of the wild-type, *E2f3*$^{+/-}$ and *E2f3*$^{-/-}$ MEFs. The cells were serum starved for 72 hours and then stimulated to re-enter the cell cycle by the re-addition of serum. Cells were harvested at regular intervals and labeled for 1 hour with [^{3}H]-thymidine to monitor DNA synthesis. Figure 3A shows the analysis of MEFs derived from two different sets of littermate embryos (H and E). The wild-type MEFs (H1 and E1)began incorporating [^{3}H]-thymidine 8-12 hours after serum stimulation and showed maximal levels of incorporation at 16-20 hours. The incorporation of [^{3}H]-thymidine by the *E2f3*$^{+/-}$ cell lines, H2 and E2, was similar. In contrast, the *E2f3*$^{-/-}$ cell lines showed significantly reduced levels and slower kinetics of [^{3}H]-thymidine incorporation. Consistent with our asynchronous studies, some of the *E2f3*$^{-/-}$ cell lines (e.g. H8 and E4) were significantly more impaired than others (e.g. H6 and E5). In each case, there was a direct correlation between the rates of proliferation and [^{3}H]-thymidine incorporation (data not shown).

We therefore conclude that the impaired proliferation of the *E2f3*$^{-/-}$ MEFs results from a defect in cell cycle progression.

There is strong evidence to suggest that E2F1 also plays a key role in the control of cellular proliferation *in vivo* (Field et al. 1996; Yamasaki et al. 1996; Pan et al. 1998; Tsai et al; 1998). We therefore examined the effects of E2F1 loss on cell cycle regulation. Strikingly, there was no detectable difference in either the level or timing of [^{3}H]-thymidine incorporation between wild-type, *E2f1*$^{+/-}$ or *E2f1*$^{-/-}$ MEFs in serum starvation/restimulation experiments (Figure 3B). This was true of multiple MEF preparations (data not shown). Consistent with this observation, we did not observe any differences in the rate of proliferation of asynchronous wild-type or *E2f1* mutant populations (data not shown). Thus, E2F1 is fully dispensable for the normal cell cycle regulation of mouse embryo fibroblasts, whereas E2F3 is rate limiting for correct cell cycle progression in response to mitogenic factors.

The reduced thymidine incorporation observed in the *E2f3*$^{-/-}$ MEFs could result from a defect in passage through the G_1/S-transition and/or a reduction in the rate of DNA synthesis. To distinguish between these two models, we followed the cell cycle re-entry of either wild-type or *E2f3*$^{-/-}$ cells at the single cell level by scoring for bromodeoxyuridine (BrdU) incorporation by immunofluorescence (Figure 3C). In the wild-type cells, BrdU incorporation was first detected 10 hours after serum re-addition. The intensity of BrdU staining continued to increase during subsequent time points, peaking at 20 hours. We detected two clear differences in the *E2f3*$^{-/-}$ cells. First, the intensity of the BrdU signal was significantly reduced, indicating a substantial reduction in the rate of BrdU incorporation and therefore of DNA synthesis. In addition, the timing

of appearance of BrdU-positive cells seemed to be delayed in the mutants relative to the wild-type cells. To quantitate this difference, we counted the number of BrdU-positive cells at each time point without scoring for the intensity of the signal (Figure 3D). At 16 hours, only 5% of the *E2f3*$^{-/-}$ cells had incorporated BrdU, compared to 20% of the wild-type cells. Even 20 hours after serum addition, the proportion of BrdU-positive cells was still lower in the *E2f3*$^{-/-}$ (19%) than in the wild-type (30%) population. These data indicate that E2F3 loss delays the initiation of DNA synthesis and dramatically reduces the rate at which this process occurs. Together, these two defects increase the time necessary to complete S-phase in a manner that is consistent with the increased doubling time of the *E2f3*$^{-/-}$ cells.

The majority of E2F-responsive genes are downregulated in the *E2f3* -/- MEFs

The timing of the cell cycle defect is consistent with the known timing of action of E2F-responsive genes. We therefore wished to determine whether the loss of E2F3 altered the expression of E2F-responsive genes and whether or not there was any correlation between the severity of the transcriptional changes and the degree of the proliferative defect. To address this issue, we compared the expression of E2F-responsive genes in a wild-type control (H1), an *E2f3*$^{+/-}$ cell line (H2: whose proliferative properties were indistinguishable from wild-type) and two *E2f3*$^{-/-}$ cell lines, one of which had a moderate cell cycle defect (H6) and one of which was dramatically impaired (H8). Parallel cell cycle fractions were used to assess [^{3}H]-thymidine incorporation (Figure 3A) or to generate RNA for northern blot analysis. The blots were normalized according to the levels of *ARPP PO*, a gene whose expression does not vary in quiescent or cycling cells (Hurford et al. 1997), and then probed for the known E2F-responsive gene

transcripts, *cyclin E, cyclin A2, cdc2, B-myb, cdc6, PCNA, RRM2. TS, DHFR* and *E2f1* (Figure 4A and data not shown). For representative genes, we quantitated the expression level based on that of the internal *ARPP PO* control (Figure 4B).

In the wild-type cells, we detected a significant cell-cycle dependent induction of most of the genes, including *cyclin E, cyclin A2, cdc2, B-myb, cdc6, PCNA* and *RRM2* (Figure 4A). [The one exception, *E2f1,* will be described in the following section]. These cell cycle regulated genes could be divided into three groups, based on whether peak expression occurred at earlier (16 hrs: *cyclin E, B-myb, cdc6* and *PCNA*), intermediate *(*16-20 hrs: *RRM2)* or later (20-24 hrs: *cyclin* A2 and *cdc2*) timepoints.

The loss of E2F3 has a profound effect on the expression of all of these cell cycle regulated, E2F-responsive genes. The severity of the transcriptional defect was most pronounced in the cell line (H8) with the severest cell cycle and proliferation defect (Figure 3A and 4A). In the H8 cells, we saw a dramatic reduction in the maximal transcript levels and peak expression was significantly delayed compared to the wild-type control (Figure 4A and B). Indeed, the cell cycle dependent induction of these genes was almost completely ablated. Similar results were observed with other E2F-responsive genes including *TS* and *TK* (data not shown). The transcriptional defects were less severe in the second $E2f3^{-/-}$ cell line, H6. In this cell line, there was little or no change in the timing of peak expression but the maximal induction of these target genes was greatly reduced. We were also able to detect some variation in the degree of down-regulation of individual target genes (Figure 4B). In some cases (e.g. *cyclin A2, cdc2, B-myb* and *RRM2*), the mRNA levels were intermediate between those observed in the wild-type and the H8, $E2f3^{-/-}$ cell line. In others (e.g *cyclin E* and *cdc6*), the degree of transcriptional

impairment approached that observed in the H8 cells. Significantly, there did not appear to be any correlation between the degree of the transcriptional defect and whether or not the gene was normally expressed at earlier, intermediate or later timepoints. Taken together, these data indicate that the loss of E2F3 significantly impairs the cell cycle dependent induction of most E2F-responsive genes and the severity of this defect correlates with the severity of the cell cycle and proliferation defect.

We also detected a significant reduction in the expression of most E2F-responsive genes in the *E2f3*$^{+/-}$ cell line, H2 (Figure 4A and B). In most cases (e.g. *cyclin A2, cdc2, B-myb* and *RRM2*), the level of expression seemed to be intermediate between that observed in the wild-type and the *E2f3*$^{-/-}$ cell lines. In contrast, expression of *PCNA* was only slightly lower than that observed in the wild-type cells, while the expression of *cyclin E* and *cdc6* much more closely resembled that seen in the *E2f3*$^{-/-}$ cell lines. These data indicate that E2F3 contributes to the correct transcriptional activation of most E2F-responsive genes in a dose-dependent manner. Importantly, the cell cycle regulation and proliferative properties of the H2, *E2f3*$^{+/-}$ cell line are indistinguishable from those of the wild-type control, H1 (Figure 3A and data not shown). Thus, changes in the levels of E2F3 can impair the transcriptional activation of most E2F-responsive genes without causing any detectable cell cycle defect. Similar results were observed in several other *E2f3*$^{+/-}$ cell lines (data not shown). This strongly suggests that the defects in cell cycle progression are a consequence, and not a cause, of the failure to induce the appropriate activation of one, or more, of these E2F-responsive genes.

E2F1 and E2F3 play distinct roles in the transcriptional regulation of MEFs.

Our transcriptional analysis detected only one known E2F-responsive gene, *E2f1*, whose expression was unaltered in the *E2f3* mutant MEFs (Figure 4A). However, contrary to the literature, the expression of this gene did not alter significantly across the cell cycle. We therefore examined the expression pattern of *E2f1* in wild-type (E1), $E2f3^{+/-}$ (E2) and $E2f3^{-/-}$ (E4) MEFs from a second set of littermate embryos (see Figure 3A). We were able to detect a significant cell cycle-dependence in the expression of *E2f1* in these wild-type MEFs (Figure 4C). Consistent with our previous studies, the expression of *B-myb* was partially impaired in the $E2f3^{+/-}$ (E2) cell line and was dramatically down-regulated in the $E2f3^{-/-}$ (E4) cell line (Figure 4C). We also observed a dramatic down-regulation of *cyclin E, cyclin A2, cdc2, cdc6, PCNA* and *RRM2* (data not shown). In contrast, we did not detect any substantive difference in the expression pattern of *E2f1* between wild-type, $E2f3^{+/-}$ or $E2f3^{-/-}$ MEFs. We therefore conclude that E2F3 is not required to maintain the normal cell cycle regulation of *E2f1* in mouse embryonic fibroblasts. This strongly suggests that the deregulation of *cyclin E, cyclin A2, cdc2, cdc6, B-myb, PCNA, RRM2, TS,* and *TK* arising from the loss of E2F3 is not an indirect consequence of changes in the level of the *E2f1* mRNA.

Considerable emphasis has been placed on understanding the specificity of target gene expression by the individual E2F family members. Therefore, we wished to establish how the loss of E2F1, the other major pRB-specific E2F, would affect the expression patterns of E2F-responsive genes. To address this issue, we conducted northern blot analysis of cell cycle fractions derived from serum starved/restimulated wild-type, $E2f1^{+/-}$ and $E2f1^{-/-}$ MEFs (Figure 5). The loss of E2F1 had no detectable effect on the cell cycle dependent expression of *cyclin A2, cdc2, cdc6, B-myb, PCNA, TS* or

RRM2. However, the expression of *cyclin E* was consistently down-regulated in the *E2f1* mutant cells. This suggests that E2F1 and E2F3 both contribute to the transcriptional regulation of the *cyclin E* gene. However, there appears to be significant specificity in the regulation of other targets. E2F3 acts, in a dose dependent manner, to determine both the timing and maximal activation of the majority of E2F-responsive genes, including *cyclin A2*, *cdc2*, *B-myb*, *cdc6*, *PCNA*, *TS*, *TK*, *DHFR* and *RRM2*. In contrast, E2F1 is fully dispensable for the correct regulation of these targets. Significantly, E2F3 is not required for the correct transcriptional regulation of the *E2f1* gene and its cell cycle dependent expression can be uncoupled from that of other E2F-responsive genes and from the G_1/S-transition.

Ectopic expression of E2F3 or E2F1 rescues the proliferation defect of *E2f3*$^{-/-}$ cells

Given these findings, we wished to establish whether we could rescue the proliferation defect of the *E2f3* mutant cells by ectopic expression of E2F3 or E2F1. To address this issue, we used recombinant replication-deficient retroviruses to re-introduce the human E2F3 and E2F1 genes into wild-type or *E2f3*$^{-/-}$ cells and then compared the growth rate of large pools of drug-resistant clones (Figure 6). The control virus had no effect on the growth rate of the *E2f3*$^{-/-}$ cells. In contrast, the expression of either E2F3 or E2F1 was sufficient to rescue the proliferation defect of the *E2f3*$^{-/-}$ cells. This confirms that the reduction in the rate of proliferation of the *E2f3*$^{-/-}$ cells is caused by the absence of E2F3 and this defect is fully reversible. At least when over-expressed, E2F1 can substitute for the loss of E2F3.

E2F3 is rate limiting for the proliferation of transformed cells

The tumor suppressive properties of pRB are thought to be largely dependent upon its ability to inhibit the transcriptional activity of the E2F transcription factors. Our data indicate that the loss of E2F3 significantly impairs the proliferation of primary cell lines. Given these findings, we wished to establish whether the absence of E2F3 would affect either the generation or proliferation of tumor cells.

Initially, we tested whether E2F3 is essential for generation of immortalized cell lines. An activated ras allele (*H-rasV12*) was introduced into wild-type and *E2f3*$^{-/-}$ MEFs with either E1A or a dominant negative p53 allele (p53R175H) using recombinant replication-deficient retroviruses. We were able to select pure populations of *E2f3*$^{-/-}$ cells that expressed either E1A + H-rasV12 or p53R175H + H-rasV12, albeit at reduced efficiency compared to the wild-type controls (data not shown). The selected wild-type and *E2f3*$^{-/-}$ populations exhibited characteristic morphologies of transformed cells (data not shown), indicating that E2F3 is not essential for the immortalization of primary mouse cells.

We next asked whether the absence of E2F3 would affect the rate of proliferation of these transformed cells. The growth rate of the wild-type and *E2f3*$^{-/-}$ transformants was compared under low density conditions, as described previously for the parental primary MEFs (Figure 2B). The expression of these oncogenes did improve the ability of the *E2f3*$^{-/-}$ cells to grow at low density (compare Figure 2B and 7). However, the E2F3-deficient cells still grew at a considerably reduced rate compared to the wild-type controls (Figure 7).

Anchorage independent growth of transformed cells correlates with tumorigenic potential *in vivo*. To examine the requirements for E2F3 in tumor cell proliferation, we assessed the ability of the wild-type and E2F3-deficient transformants to grow in soft agar. After five days in the semisolid media, the wild-type cells formed discrete foci that increased in size over time (figure 7 and data not shown). Significantly, the *E2f3*$^{-/-}$ cells formed far fewer foci that each contained significantly fewer cells than their wild-type controls (figure 6 and data not shown). This was true regardless of whether the cells were transformed with E1A and H-rasV12 or p53R175H and H-rasV12. Based on these findings, we conclude that E2F3-deficiency does not prevent the transformation of primary murine cells and the subsequent ability of these cells to grow in soft agar. However, the absence of E2F3 compromises the ability of these cells to proliferate. Taken together, our data indicate that E2F3 is rate-limiting for the proliferation of both primary and tumor cells.

DISCUSSION

The role of E2F3 in the control of cellular proliferation

We have used cell lines derived from *E2f3* mutant mice to investigate the role of E2F3 in cell cycle regulation. These studies show that the loss of E2F3 significantly reduces the rate of cellular proliferation of both primary and transformed cell lines. This is caused by an increase in doubling time that results from defects in the initiation and rate of progression of DNA synthesis. This observation is highly consistent with the prior report that anti-E2F3 antibodies can inhibit rat embryonic fibroblasts from entering S-phase (Leone et al. 1998). In addition, our E2F3 mutant cells have a major defect in the regulation of E2F-responsive gene transcription. In the $E2f3^{-/-}$ cells we see a dramatic impairment of the transcriptional activation of many E2F-responsive genes. The degree of this transcriptional defect correlates closely with the severity of the proliferation defect indicating that these two phenotypes are closely linked. In contrast, in the majority of the $E2f3^{+/-}$ cell lines we see a reduction in the maximal activation of the same panel of E2F-responsive genes without any detectable cell cycle or proliferation defects. This observation yields two important conclusions. First, E2F3 contributes to the transcriptional regulation of many E2F-responsive genes in a dose-dependent manner. Second, cells can tolerate limited reduction in the expression of these targets without any deleterious consequences but, once expression drops below a critical threshold, there is a direct correlation between the level of expression and the rate of proliferation. By extension of this logic, we conclude that E2F3 plays a key role in regulating the

expression of one, or more, target genes that determine the rate of initiation and progression of DNA synthesis.

E2F3 plays a key role in mediating the transcriptional activation of most E2F-responsive genes.

Our studies yield considerable insight into the general mechanisms of regulation of individual E2F-responsive genes. Based on a combination of *in vivo* footprinting and promoter mapping experiments, other studies have concluded that many E2F-responsive genes, including *B-myb*, *cdc2*, *cyclin A* and *E2f1*, will be primarily regulated by the binding of repressive, pocket protein•E2F complexes during the G_0/G_1 stage of the cell cycle (Dalton 1992; Lam and Watson 1993; Neuman et al. 1994; Tommasi and Pfeifer 1995; Huet et al. 1996; Zwicker et al. 1996; Le Cam et al. 1999). In this paper, we have shown that the mutation of E2F3 has no effect on the regulation of E2F-responsive genes during G_0/G_1 but it inhibits the normal, cell cycle-dependent induction of these targets in a dose dependent manner. This indicates that activating E2F complexes must play a key role in mediating the induction of these genes at the G_1/S-transition. It is unclear why previous approaches have failed to appreciate the importance of activating E2F complexes. Since *in vivo* footprinting requires site-occupancy within a high proportion of the cell population, it is biased towards the detection of stable complexes. It is therefore possible that the transcriptionally active E2F-3 complexes bind to the promoter in a narrow window of time that cannot be detected by existing cell synchronization methods. It is less easy to explain why the promoter mapping studies detect repressive and not activating E2F complexes but this could be attributed to differences in chromatin assembly on transiently transfected reporters versus the endogenous promoter. Clearly,

our data do not refute the importance of repression in the regulation of E2F-responsive genes but they provide strong genetic evidence that activation by E2F3 plays a major role in mediating the cell cycle-dependent induction of these targets.

Identification of E2F3 downstream target genes

It is widely believed that the different biological properties of pRB family members are mediated through their ability to regulate different E2F family members with distinct biological properties. This is supported by the finding that different subsets of E2F-responsive genes are deregulated in $Rb^{-/-}$ or $p107^{-/-}; p130^{-/-}$ mutant MEFs (Herrera et al. 1996; Hurford et al. 1997). This has raised considerable interest in establishing the target specificity of the individual E2F complexes. Our studies provide strong genetic evidence that the normal cell cycle dependent activation of many known E2F-responsive genes is dependent upon E2F3. In particular, we have identified a number of genes, including *cyclin E, cyclin A2, cdc2, cdc6, B-myb* and *RRM2*, whose transcription is downregulated in $E2f3^{+/-}$ cell lines that have no detectable cell cycle defect. This strongly suggests that the altered expression of these genes occurs independently of any changes in the timing of the G_1/S transition.

We can subdivide these target genes into two distinct subgroups. The expression of genes in the first subgroup, which includes *cyclin A2, cdc2, B-myb* and *RRM2*, is directly proportional to the *E2f3* gene dosage. In contrast, genes in the second subgroup, *cyclin E* and *cdc6*, appear particularly sensitive to any change in the levels of E2F3. Indeed, mutation of a single *E2f3* allele impairs the cell cycle-dependent expression of these genes almost as efficiently as the complete loss of E2F3. Thus, expression of *cyclin*

E and *cdc6* seems to require a critical threshold level of free E2F activity that is close to the maximal levels present in these cells and higher than the levels required to activate expression of *cyclin A2, cdc2, B-myb* and *RRM2.* Significantly, the peak expression of *cyclin E* and *cdc6* occurs earlier in the cell cycle than that of many of the other E2F-responsive targets. This suggests that accumulation of critical threshold levels of E2F3 cannot fully account for the differential timing of expression of these genes.

Our data strongly suggest that the loss of expression of one, or more, of the E2F3 –regulated genes impairs the ability of the cells to proliferate. It is clearly important that we identify the rate-limiting gene(s). Since the absence of E2F3 significantly reduces the rate of DNA synthesis, it is tempting to speculate that at least one of the critical targets may be directly involved in the DNA replication process. Indeed, several of the E2F3-dependent genes are known to be required for the initiation of DNA replication (*cdc6;* Stillman 1996*)* or the maintenance of the nucleotide pools (*RRM2* and *TS*) and there are many other candidates, including *DNA polymerase α, orc1* and *mcm 2-7* (Stillman 1996), whose expression we have yet to analyze.

We have also identified E2F-responsive genes that do not appear to be directly regulated by E2F3. Since we detect little difference in the expression of PCNA between the wild-type and the *E2f3*$^{+/-}$ cell lines, it is unclear whether the reduced PCNA expression in *E2f3*$^{-/-}$ cells is a direct consequence of E2F3 loss or an indirect consequence of changes in cell cycle regulation. More striking is the regulation of *E2f1.* Our studies indicate that the expression pattern of this gene is not altered in any of the *E2f3* mutant cell lines despite the presence of a well-documented E2F site in the *E2f1* promoter (Hsiao et al. 1994; Johnson et al. 1994; Neuman et al. 1994). This suggests that E2F3 is not

required to maintain the correct cell cycle regulation of *E2f1*. Consequently, at least in the absence of E2F3, the expression of this gene must be mediated by other E2F family members or in an E2F-independent manner. Most importantly, our data indicate that *E2f1* expression continues to be activated normally despite a substantial delay in the timing of the initiation of S-phase. This result indicates that *E2f1* expression can be uncoupled from the G_1/S-transition and from the induction of most other E2F-responsive genes. These data strongly suggest that E2F1 and E2F3 function independently of one another.

Target specificity of individual E2F family members

We have shown that E2F3 plays a key role in mediating the cell cycle-dependent induction of most E2F-responsive genes in mouse embryonic fibroblasts. Clearly, the remaining E2F family members are unable to substitute for the loss of E2F3. This is true even in the presence of E1A, which mediates the release of all E2F•DP complexes through the sequestration of pRB-family members. These observations raise clear questions about the role of other E2F family members. Do they have distinct transcriptional targets or is there some degree of functional redundancy? To address this question, we directly compared the consequences of E2F3 and E2F1 deficiency. We selected E2F1 for a number of reasons. First, E2F1 and E2F3 are the major pRB-specific E2Fs. Second, the analyses of mutant mouse strains show that E2F1 makes a significant contribution to the inappropriate proliferation arising from the functional inactivation of pRb (Pan et al. 1998; Tsai et al. 1998; Yamasaki et al. 1998). Third, the availability of *E2f1* mutant mice (Yamasaki et al. 1996) allowed us to compare the roles of E2F1 and E2F3 within a common cell type. Finally, our data indicate that the *E2f1* gene is

regulated independently of E2F3. Our analyses of *E2f1* mutant MEFs indicate that the loss of E2F1 has no detectable effect on either the cell cycle regulation or the proliferative capacity of primary murine fibroblasts. Similarly, E2F1 is not required for the correct cell cycle expression of many E2F-responsive genes, including most of those affected by E2F3 loss (*cyclin A2, cdc2, cdc6, B-myb* and *RRM2).* However, the loss of E2F1 causes a down-regulation in the levels of *cyclin E* that are comparable to that observed in the E2F3 mutant cells. Consistent with this finding, Wang et al. (1998) have also reported that the cell cycle dependent expression of cyclin E is impaired in E2F1-deficient cells. Since there is no proliferation defect in the *E2f1* mutant MEFs, the downregulation of the *cyclin E* mRNA levels cannot fully account for the cell cycle defects arising in the $E2f3^{-/-}$ MEFs.

The differential regulation of E2F-responsive genes in *E2f1* and *E2f3* mutant cells supports two alternative models of E2F function. First, E2F1 and E2F3 could have very different biological properties that result from differences in target gene regulation. In this model, E2F3 acts as the "work-horse" to mediate the cell cycle-dependent activation of the key components of the cell cycle control and DNA replication machinery in response to mitogenic signals. In contrast, E2F1 acts primarily in response to inappropriate signals, such as DNA damage or uncontrolled proliferation, as a "surveillance" mechanism. This model is supported by the finding that E2F3 is critical for the normal proliferation of cell lines and the normal development and viability of E2F3-deficient mice. In contrast, E2F1 seems largely dispensable for normal cellular proliferation and development but there is strong evidence to support its role in apoptosis. First, E2F1, but not the other E2Fs, induces apoptosis when overexpressed in quiescent

cells (DeGregori et al. 1997). Second, *E2f1*$^{-/-}$ mice exhibit a defect in thymocyte apoptosis and are tumor prone (Field et al. 1996; Yamasaki et al. 1996). Finally, loss of E2F1 causes a dramatic reduction in the level of apoptosis arising from the functional inactivation of pRB (Pan et al. 1998; Tsai et al. 1998).

The second model proposes that E2F1 and E2F3 regulate common target genes but their differential biological properties result from differences in their relative expression levels. Since E2F3 is expressed at higher levels than E2F1 in MEFs, the loss of this protein brings the levels of free transcriptionally active E2F below the critical threshold that is required for the correct regulation of most E2F responsive genes. In contrast, the reduction in free E2F activity arising from the loss of E2F1 is only sufficient to impair the expression of a single gene, *cyclin E.* This model is entirely consistent with our conclusion that *cyclin E* is extremely sensitive to the levels of activating E2F. Moreover, at least when over-expressed, E2F1 can rescue the proliferation defect in the E2F3 -/- MEFs in a similar manner to E2F3.

Understanding the role of E2F3 in tumorigenesis

The retinoblastoma protein is functionally inactivated in most, if not all, human tumors (Weinberg et al. 1992). E2F3 is one of three E2F family members that are specifically regulated by this tumor suppressor (Lees et al. 1993). We have now shown that E2F-3 regulates the expression of genes that determine the rate of proliferation of both primary and tumor cell lines. These observations suggest that E2F3 will make a major contribution to the inappropriate proliferation resulting from the loss of pRB. Given this hypothesis, it will be important to establish whether the loss of E2F3 alters the viability

of *Rb* homozygous mutant embryos or the rate of tumor formation in *Rb* heterozygous mutant mice. This will allow us to establish how E2F3 contributes to tumorigenesis *in vivo* and will yield critical insight into the relative roles of E2F1 and E2F3.

MATERIALS AND METHODS

Construction of E2f3 targeting vector

Overlapping mouse *E2f3* genomic clones containing the *E2f3* cyclin A binding domain, DNA binding domain and the dimerization domain exons were isolated from a 129/Sv mouse library using standard techniques. A 0.9kb *Hind*III fragment containing the cyclin A binding domain was subcloned into pBKS. An in frame STOP codon was inserted after the third codon of the *E2f3* cyclin A binding domain by inserting an engineered *Xba*I-*Pvu*II linker. A 750 bp *Kpn*I-*Xba*I fragment was then transferred into pPNT (Tybulewicz et al. 1991) and a 3.1 kb *Kpn*I genomic fragment containing additional 5' sequences was added. The targeting vector, *E2f3-neo,* was completed by subcloning a 3.4 kb *EcoR*I- *EcoR*V 3' genomic fragment into the *Not*I and *Xho*I sites using linkers.

Generation of targeted ES cells and E2f3 deficient mice

D3 ES cells were electroporated with 50 μg of *Not*I linearized *E2f3-neo* and selected for resistance to G418 (300 μg/ml) and Gancyclovir (0.5 μg/ml). DNA from double resistant ES cell clones was digested with *Bgl*II and analyzed by southern blotting using a 720 bp *Rsa*I DNA fragment as the 5' probe. Two independent electroporations yielded 29 clones with a novel 6.5 kb band corresponding to a correctly targeted 5' end (WT, 9.5 kb). DNA from these clones was digested with *Xba*I and probed with a 650 bp *EcoR*I-*Kpn*I 3' fragment (MUT, 9 kb versus WT, 11 kb) and then a 450 bp *Pst*I-*Hind*III *neo* fragment. 22/29 ES clones contained a single integration of the *E2f3* targeting vector

that had undergone correct homologous recombination on each side of the *neo* cassette. These ES cell clones were injected into 3.5 day C57BL/6 blastocysts and the resulting chimerics were mated to C57BL/6 females. One clone from each electroporation (ES clones F3-1-1 and F3-2-13) transmitted the mutation through the germline. The targeted *E2f3* allele was detected in agouti pups using Southern blotting of tail DNA as described above. PCR of mouse ear punch DNA was then used for subsequent genotyping using the common primer 5'-GTATCTGGGAAACACAAGGAGGTG, the wild-type *E2f3*-specific primer 5'-GGTACTGATGCCACTCTCGCC, and the targeting vector specific primer, 5'-GCTCATTCCTCCCACTCATGATC.

MEF preparation

E2f3$^{+/-}$ females were crossed with *E2f3*$^{+/-}$ males and embryos were dissected 13.5 days after detection of vaginal plugs. The head and internal organs were removed and the embryos were minced and incubated in trypsin for 30 min at 37°C. The cells were resuspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin, 50 μg /ml streptomycin, and 2mM L-glutamine. Fetal livers and/or yolk sacs were used for PCR genotyping.

High and low density growth experiments

For the high density experiments, the MEFs were plated at $2x10^5$/6 cm dish. Cells were counted as they reached confluence and replated at $2x10^5$ cells/6 cm dish. For low density experiments, MEFs were plated at a density of $1x10^5$ cells/10 cm dish and their growth rate was monitored by daily counting for 10 days. For the E2F3 and E2F1 rescue

experiments, transduced wild-type or *E2f3* mutant cells were plated at $2x10^5$ per 6 cm dish, and their growth rate was monitored for 4 days. Transformed cells were plated at $5x10^4$ per 10 cm dish and counted daily for 6 days.

Serum starvation and release experiments

Passage 4 MEFs were plated in triplicate at $2x10^5$/3.5 cm dish. After 48 hours, the cells were washed twice with PBS and then incubated in DMEM containing 0.1% FCS for 72 hours. The cells were then fed with DMEM containing 10% FCS. For each time point, the cells were incubated with 5 μCi [^{3}H]-thymidine for 1 hour at 37°C, washed with PBS and harvested. [^{3}H]-thymidine incorporation was quantitated as described (Moberg et al. 1996). For bromodeoxyuridine (BrdU) incorporation experiments, cells were plated onto coverslips. At each timepoint, the cells were incubated in media containing 3 mg/ml BrdU and 0.3 mg/ml fluorodeoxyuridine (FdU) for 2 hours at 37°C (Sigma). The cells were fixed for 15 min in 2% paraformaldehyde and permeabilized with PBS/0.25% Triton X-100. After denaturing the DNA for 10 min in 1.5N HCl, the cells were incubated with mouse anti-BrdU antibodies (Beckton-Dickinson, 1:50) for 30 min and then with FITC-anti mouse antibodies (Capel, 1:1000) for 30 min. The coverslips were washed four times, incubated with 4',6'-diamidino-2-phenylindole (DAPI) (0.1 mg/ml) for 5 min, washed and mounted on glass slides with Vectashield (Vector).

Northern blot analysis

Passage 4 MEFs were plated onto 15 cm dishes at $3x10^6$ cells/dish and then serum starved as described above. At each time point, the cells were pelleted and RNA purified using the Ultraspec™ RNA isolation system (Biotex Laboratories, Inc). The RNA was denatured and separated on gels containing 1% agarose, 6% formaldehyde and 1xMOPS buffer pH 7.0. The RNA was transferred to Hybond™-N nylon membranes (Amersham), hybridized in ExpressHyb solution (Clontech) and washed twice in 2xSSC, 0.1 % SDS for 30 min at 65°C. The cDNA probes were labeled using the Prime-It II-kit (Stratagene) with 100 μCi of [α-^{32}P] dCTP. The amount of RNA used for each timepoint was determined by probing a test Northern with the *ARPP PO* control. Subsequent Northerns were then probed with full length cDNAs for *B-myb*, *cdc2*, *cdc6*, *cyclin A2*, *cyclin E*, *RRM2*, *PCNA* or *cyclin D1* or a partial *E2F-1* cDNA fragment (nt 524-1388) and then re-probed for *ARPP PO*. The expression level of each gene was quantitated by phosphorimager analysis and normalized to the levels of *ARPP PO*.

Western blot and gel retardation assays

Western blotting and gel retardation assays were performed as described previously (Moberg et al. 1996) using 100 μg or 30 μg of whole cell lysates respectively. Western blotting was conducted using anti-E2F3 (Santa Cruz sc-878, 1:1000) and an HRP-coupled anti-rabbit antibody (Amersham, 1:5000). Gel retardation assays were performed in the absence or presence of sodium deoxycholate (DOC) as described (Moberg et al. 1996) using antibodies against E2F1(KH95 and KH20), E2F2 (LLF2-1), E2F3 (Santa Cruz sc-878x), E2F4 (LLF4-1) or E2F5 (Santa Cruz, sc-1083x).

Retroviral mediated gene transfer and soft agar assay

pBabe-E2F3 and pBabe-E2F1 were generated by subcloning human E2F3 and E2F1 into the pBabe vector. The retroviral mediated transfer was conducted as described by Serrano et al. (1997) except the infected cells were grown for 2 days prior to selection with 2 μg/ml puromycin (pBABE-E2F3, pBABE-E2F1 and pBABE-H-rasV12) or 75 μg/ml hygromycin (pWZL-E1A and pWZL-p53R175H). For soft agar assays, 6 cm dishes were coated with 0.5% low melting point (LMP) agarose (Gibco BRL) in DME containing 10% FCS. $5x10^4$ cells were resuspended in 0.3% LMP agarose plus DME with 10% FCS and grown on the coated dishes for 1-2 weeks.

ACKNOWLEDGEMENTS

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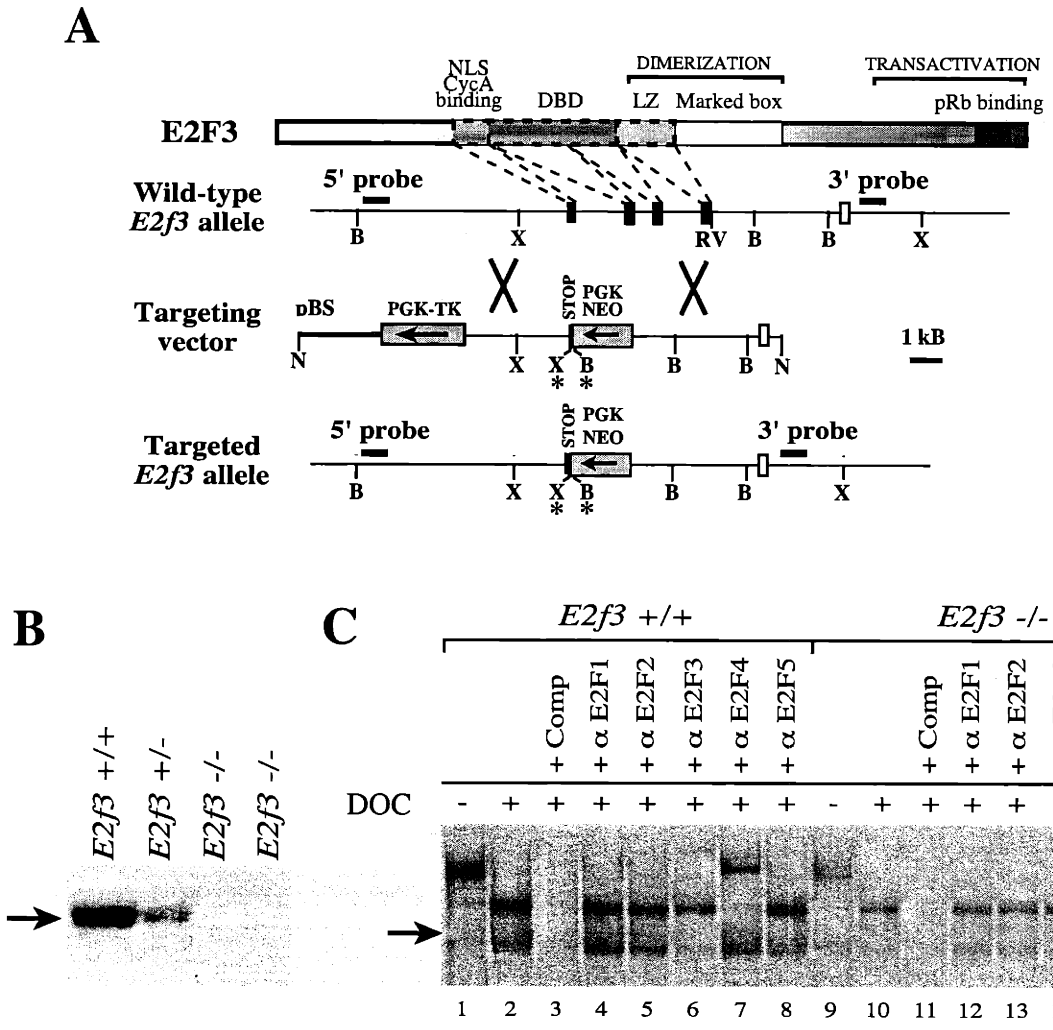
TABLE 1. *E2f3* is essential for full viability

	E2f3 +/+	*E2f3 +/-*	*E2f3 -/-*
Number of pups*	**80**	**174**	**19**
Expected ratio	**1**	**2**	**1**
Observed ratio	**1**	**2.2**	**0.24**

* Progeny arising from an intercross of E2f3 heterozygous mutant mice in the mixed (C57BL/6 x 129/sv) genetic background


A
NLS
CycA
binding
DBD
DIMERIZATION
LZ
Marked box
TRANSACTIVATION
pRb binding
E2F3
Wild-type
E2f3 allele
5' probe
3' probe
B
X
RV
B
B
X
Targeting
vector
pBS
PGK-TK
STOP
PGK
NEO
1 kB
N
X
X
B
B
B
N
Targeted
E2f3 allele
5' probe
STOP
PGK
NEO
3' probe
B
X
X
B
B
B
X
B
E2f3 +/+
E2f3 +/-
E2f3 -/-
E2f3 -/-
C
E2f3 +/+
E2f3 -/-
+ Comp
+ α E2F1
+ α E2F2
+ α E2F3
+ α E2F4
+ α E2F5
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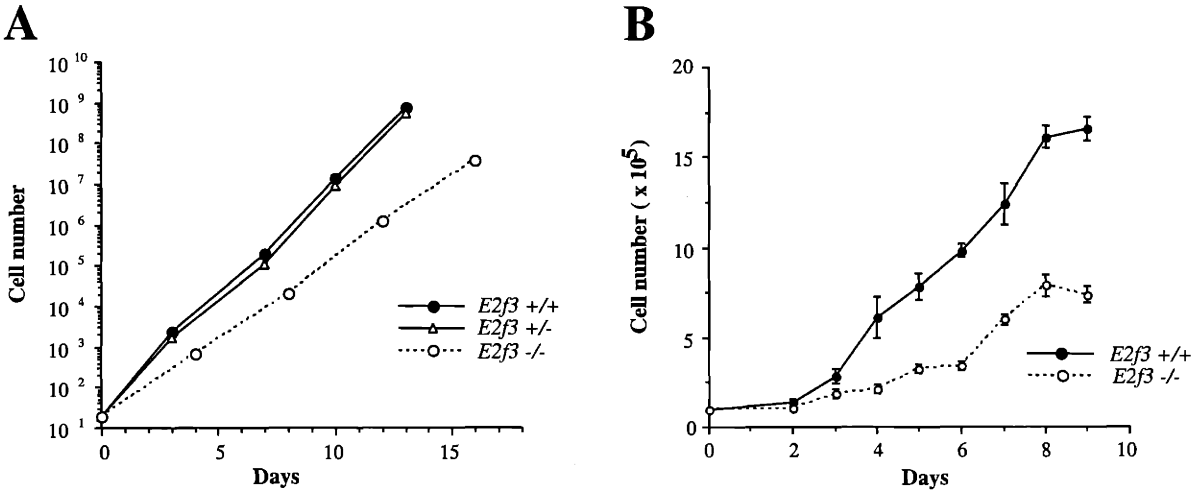
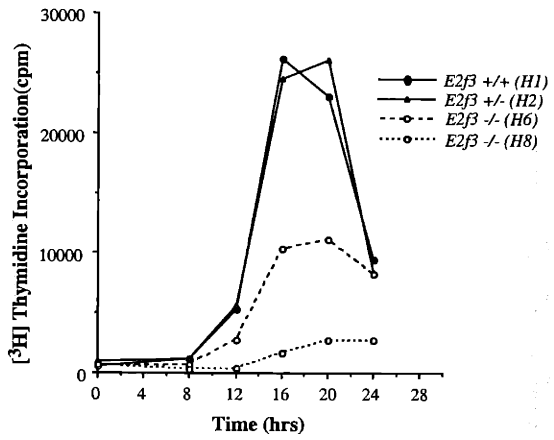
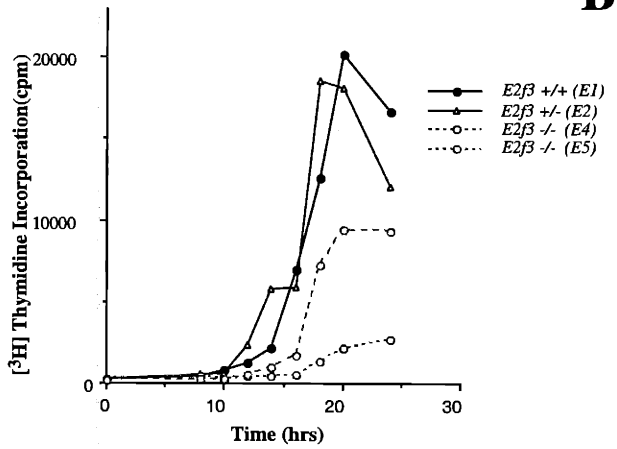
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 2


A
Cell number
Days
E2f3 +/+
E2f3 +/-
E2f3 -/-
B
Cell number (x 10^5)
Days
E2f3 +/+
E2f3 -/-

A


30000
20000
10000
0
[3H] Thymidine Incorporation(cpm)
0 4 8 12 16 20 24 28
Time (hrs)
E2f3 +/+ (H1)
E2f3 +/- (H2)
E2f3 -/- (H6)
E2f3 -/- (H8)


20000
10000
0
[3H] Thymidine Incorporation(cpm)
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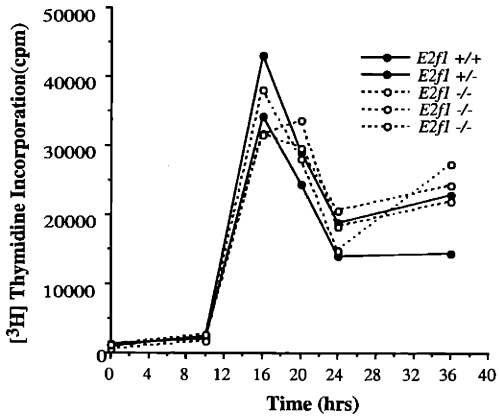
Time (hrs)
E2f3 +/+ (E1)
E2f3 +/- (E2)
E2f3 -/- (E4)
E2f3 -/- (E5)

B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30000
20000
10000
0
[3H] Thymidine Incorporation(cpm)
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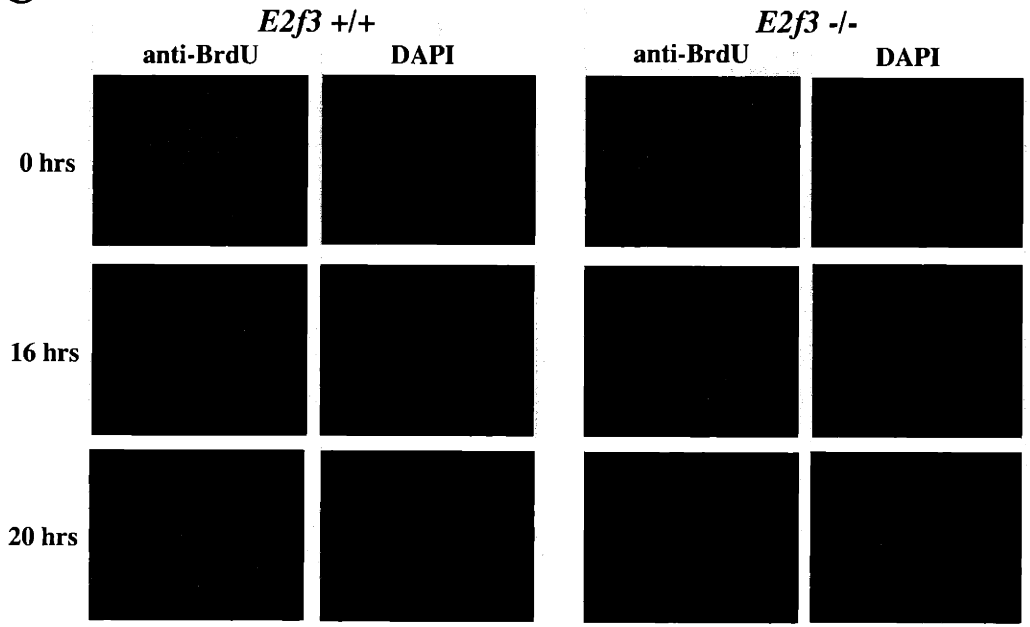
Time (hrs)
E2f1 +/+
E2f1 +/-
E2f1 -/-
E2f1 -/-
E2f1 -/-

C


E2f3 +/+
anti-BrdU
DAPI
E2f3 -/-
anti-BrdU
DAPI
0 hrs
16 hrs
20 hrs

D

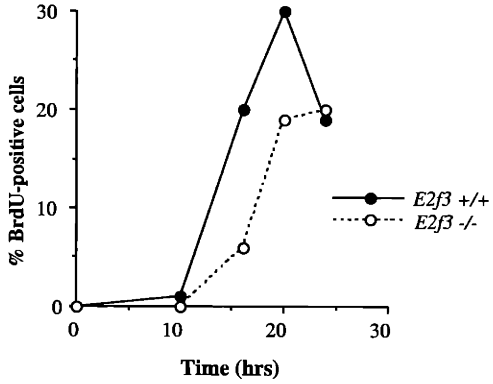

30
20
10
0
% BrdU-positive cells
0 10 20 30
Time (hrs)
E2f3 +/+
E2f3 -/-

Figure 4

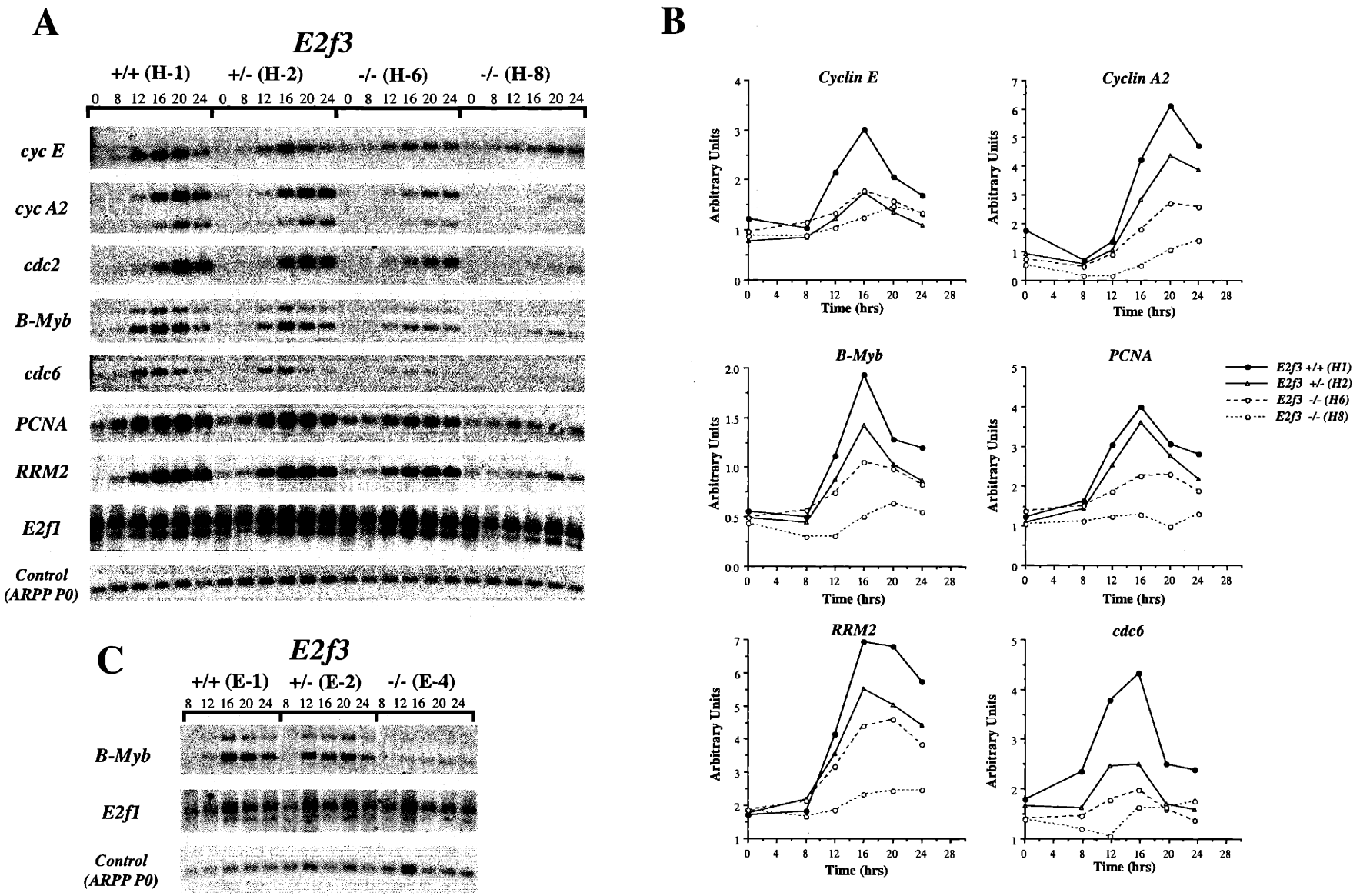
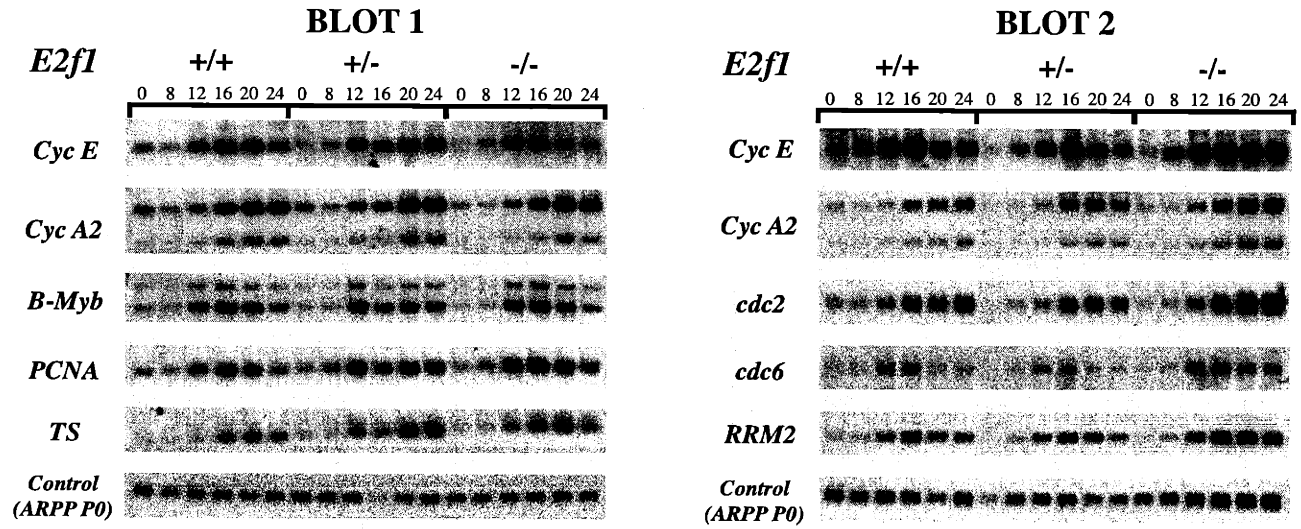

A
E2f3
+/+ (H-1)
+/- (H-2)
-/- (H-6)
-/- (H-8)
0 8 12 16 20 24 0 8 12 16 20 24 0 8 12 16 20 24 0 8 12 16 20 24
cyc E
cyc A2
cdc2
B-Myb
cdc6
PCNA
RRM2
E2f1
Control (ARPP P0)
B
Cyclin E
Cyclin A2
B-Myb
PCNA
RRM2
cdc6
Arbitrary Units
Time (hrs)
E2f3 +/+ (H1)
E2f3 +/- (H2)
E2f3 -/- (H6)
E2f3 -/- (H8)
C
E2f3
+/+ (E-1)
+/- (E-2)
-/- (E-4)
8 12 16 20 24 8 12 16 20 24 8 12 16 20 24
B-Myb
E2f1
Control (ARPP P0)

Figure 5


BLOT 1
E2f1
+/+
+/-
-/-
0 8 12 16 20 24 0 8 12 16 20 24 0 8 12 16 20 24
Cyc E
Cyc A2
B-Myb
PCNA
TS
Control (ARPP P0)
BLOT 2
E2f1
+/+
+/-
-/-
0 8 12 16 20 24 0 8 12 16 20 24 0 8 12 16 20 24
Cyc E
Cyc A2
cdc2
cdc6
RRM2
Control (ARPP P0)

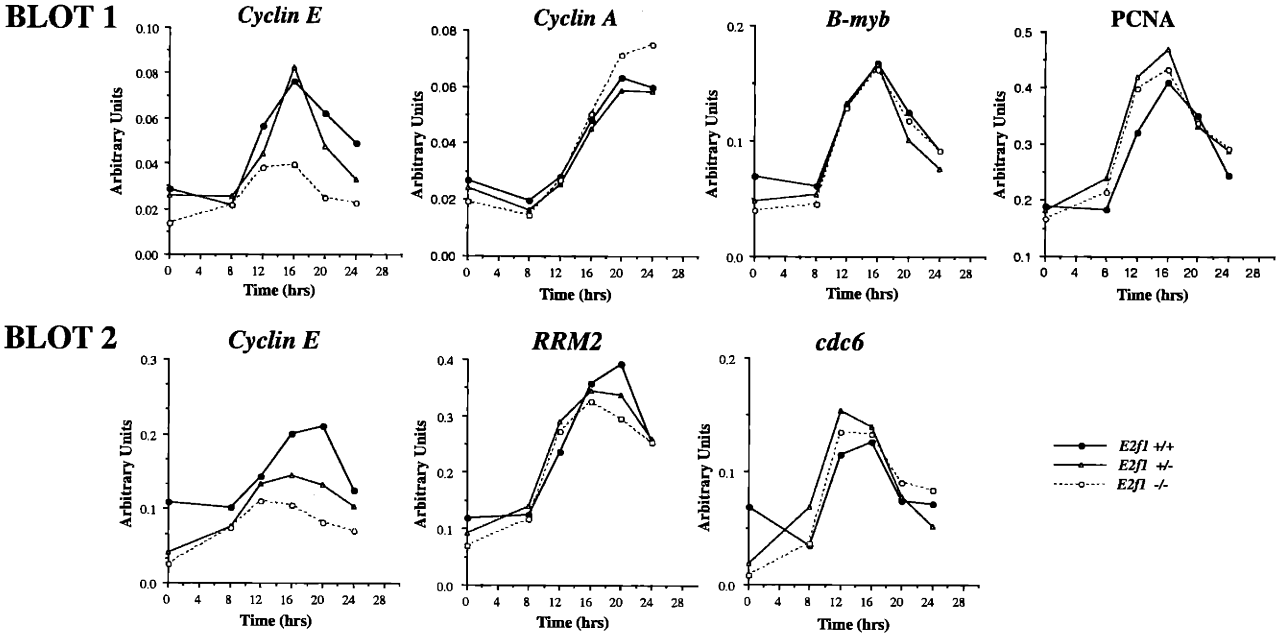

BLOT 1
Cyclin E
Cyclin A
B-myb
PCNA
Arbitrary Units
Time (hrs)
BLOT 2
Cyclin E
RRM2
cdc6
Arbitrary Units
Time (hrs)
E2f1 +/+
E2f1 +/-
E2f1 -/-

Figure 6

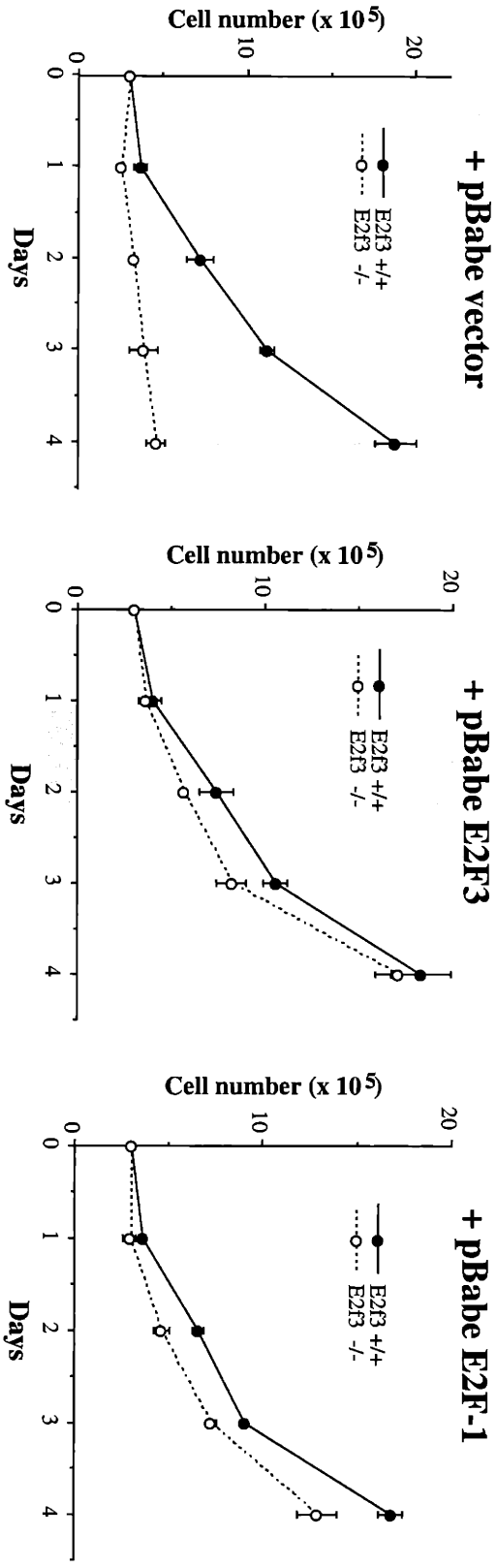

+ pBabe vector
Cell number (x 10^5)
Days
E2f3 +/+
E2f3 -/-
+ pBabe E2F3
Cell number (x 10^5)
Days
E2f3 +/+
E2f3 -/-
+ pBabe E2F-1
Cell number (x 10^5)
Days
E2f3 +/+
E2f3 -/-

Figure 7

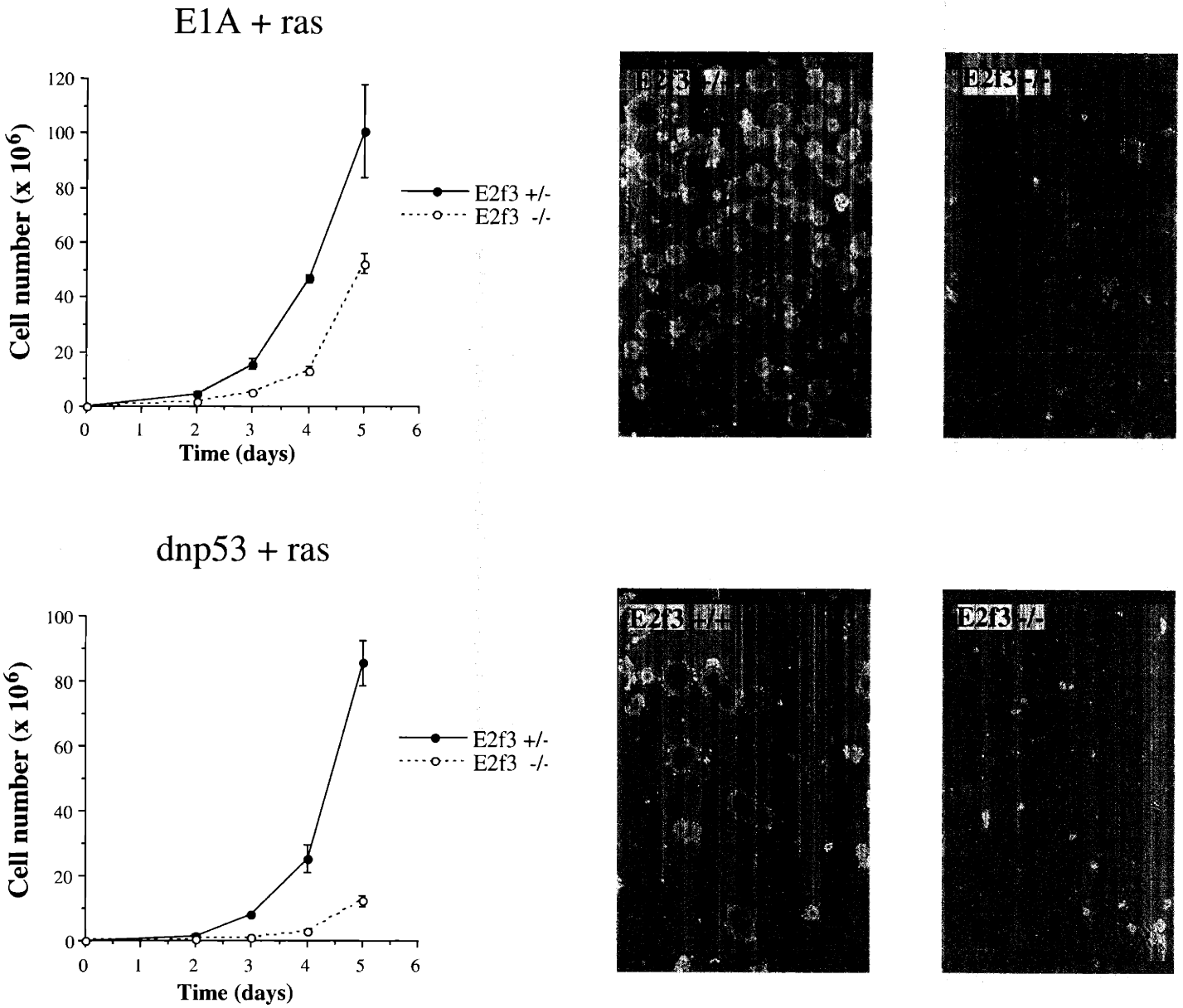

E1A + ras
Cell number (x 10^6)
Time (days)
E2f3 +/-
E2f3 -/-
dnp53 + ras
Cell number (x 10^6)
Time (days)
E2f3 +/-
E2f3 -/-

FIGURE LEGENDS

Figure 1. E2F3 does not affect the expression of other E2F species. (A) The *E2f3* gene was inactivated in ES cells using the targeting construct shown. This strategy introduces an in-frame termination codon upstream of the nuclear localization signal (NLS) of E2F3 and replaces the exons encoding the NLS, DNA binding (DBD) and leucine zipper (LZ) domains (shown in black) with a neomycin resistance gene. (B) Western blot analysis was performed on whole cell extracts generated from MEFs derived from the progeny of $E2f3^{+/-}$ crosses. E2F3 is indicated (see arrow). (C) To examine how E2F3 loss affects other E2F species, gel shift analysis was performed in the absence or presence of sodium deoxycholate (DOC) which releases the free E2F•DP complexes from the associated pRB family members. The free DP•E2F3 complex is indicated (see arrow).

Figure 2. $E2f3^{-/-}$ cells have a proliferation defect. Passage 4 MEFs derived from wild-type and *E2f3* mutant littermates were cultured under high (A) or low (B) density conditions as described in the methods.

Figure 3. $E2f3^{-/-}$ cells have defects in the initiation and progression of DNA synthesis. (A) Wild-type and *E2f3* mutant MEFs from two different MEF preparations (H and E) were synchronized by serum starvation and their cell cycle re-entry properties assayed using [^{3}H]-thymidine incorporation. (B) MEFs derived from a wild-type, an $E2f1^{+/-}$ and three $E2f1^{-/-}$ littermate embryos were analyzed as in (A). (C and D) Wild-

type and *E2f3* $^{-/-}$ cells were plated on coverslips and synchronized by serum starvation. At each time point after serum addition, DNA synthesis was monitored by assaying for bromodeoxyuridine (BrdU) incorporation.

Figure 4. E2F3 is required for the mitogen induced, transcriptional activation of most known E2F-responsive genes. Wild-type, *E2f3* mutant MEFs were synchronized by serum starvation. At each time point, RNA was isolated and equal amounts of RNA were subjected to northern blot analysis to determine the pattern of expression of E2F-responsive genes (A and C). (B) The expression level of selected genes shown in (A) was quantitated using phosphorimager analysis and normalized to the *ARPP P0* control.

Figure 5. The loss of E2F1 does not affect the expression of most E2f-responsive genes. Wild-type, heterozygous or homozygous *E2f1* mutant MEFs were synchronized by serum starvation/readdition and northern blot analysis was performed at various cell cycle stages. The expression levels of selected genes was quantitated using phosphorimager analysis and normalized to the *ARPP P0* control.

Figure 6. The proliferation defect of the *E2f3 -/-* cells can be rescued by the ectopic expression of E2F3 or E2F1. Wild-type or *E2f3* mutant MEFs were infected with either control, E2F3, or E2F1 expressing retroviruses. After four days selection, the cells were plated at equal densities and their growth rates monitored.

Figure 7. E2F3-deficiency impairs the proliferation of transformed cell lines. Wild-type or *E2f3*$^{-/-}$ MEFs were infected with retroviruses expressing an activated *ras* allele (*H-rasV12*) and either *E1A* or a dominant negative *p53* allele, *p53 R175H*. After selection, cells were plated at equal densities and their proliferation rates were monitored by daily counting. To assess anchorage-independent growth, equal numbers of transformed cells were plated in 0.3% low melting point agarose. Representative wild-type and *E2f3* mutant fields are shown.

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